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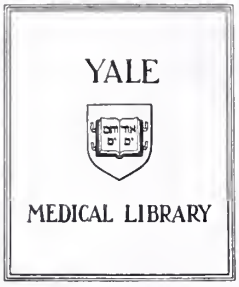
# WISKOTT-ALDRICH SYNDROME:

## A STUDY OF THE CARRIER STATE USING POLYMORPHOUS GLUCOSE-6-PHOSPHATE DEHYDROGENASE AS A GENETIC MARKER

W. James Gealy, Jr.

1979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# WISKOTT-ALDRICH SYNDROME:

# A STUDY OF THE CARRIER STATE USING POLYMORPHOUS GLUCOSE-6-PHOSPHATE DEHYDROGENASE AS A GENETIC MARKER

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February, 1979
New Haven, Connecticut

A thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine

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To my best friend Frank, who thought I was nuts ....

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# INTRODUCTION

Much of what we know about the human immune system has been discovered by studying patients with defective immunity and comparing their defects with the systems of normal patients. One of the more fascinating of these defective immune systems is seen in the Wiskott-Aldrich syndrome (WAS), a sex-linked recessive and usually fatal disorder characterized by eczema, abnormal platelets, recurrent infections, and broad defects in humoral and cell-mediated immunity. In an attempt to better understand this disorder numerous attempts have been made to elucidate the cell line responsible for the immune defects. At one time or another, however, every hematogenous cell line except the erythrocyte has been suggested as a possible contributor. Except for the fact that the disease can be cured by complete bone-marrow stem cell replacement, the metabolic and cellular basis for the disorder remain unknown.

In an attempt to clarify much of the immunologic work done on WAS patients in the past, this thesis presents an angle of attack completely different from any yet applied to this disorder. A study of the X-chromosome inactivation patterns in the carriers of this disorder was undertaken in an attempt to locate possible cellular defects responsible for the expression of the syndrome. Results suggesting the location of this cellular defect as well as a possible method for detecting the carrier state will be

presented. The possible application of this technique to the study of other sex-linked recessive immunologic diseases will be discussed as well.

## BRIEF HISTORICAL SKETCH

A syndrome of thrombocytopenia, eczema, and repeated infections was first described in a single patient by Wiskott[1] in 1937. The second description of this syndrome appeared seventeen years later in 1954 when Aldrich et al[2] noted the sex-linked recessive nature of this clinical triad in a large family. Huntley and Dees[3] observed the presence of eosinophilia and milk allergy and were the first to suggest that splenectomy was followed by increased susceptibility to infections.

Defects in immunologic function were demonstrated by many. West et al[4] first described decreased serum IgM and elevated IgA levels. Krivit and Good[5] showed that these WAS children had deficient antibody production in response to certain bacterial antigens. Deficient cell-mediated immunity was described by Cooper et al[6]. Absence of isohemagglutinins was reported by Krivit et al[7] and Pearson et al[8] who also suggested that thrombocytopenia was due to decreased platelet survival time.

Cooper et al[9] suggested that this disorder was progressive and that the immune response of these patients deteriorated with age. Blaese et al[10] felt that the defects in both humoral and cellular immunity could be explained by a defect in antigen processing or recognition. Cooper[11] postulated a defect in a B-lymphocyte subline whereas bone marrow transplantation work by Parkman et al[12] suggested a T-lymphocyte defect.

Abnormalities in other cell lines have been shown as well, although to a lesser degree.

The clinical course of this disorder has been well described by Steihm and Fulginiti[13]. Those patients with the more severe forms of the disorder succumb to hemorrhage or severe infections within the first few years of life whereas those with the milder forms have been reported to survive at least into their mid-twenties. The initial manifestations of the syndrome are thrombocytopenia, which is often present at birth, and eczema, which invariably develops within the first four to six months of life. As the WAS child ages the bleeding problems often subside whereas the eczema frequently becomes severe and may at times be difficult to distinguish from superficial infection of the skin. Recurrent bacterial and viral infections make their life quite miserable. Pneumococci, staphylococci, cytomegalovirus, herpes simplex, and Pneumocystis carinii are some of the more common infecting organisms. Hematologic and immunologic support of these children often prolongs life. Many however often go on to develop lymphoreticular tumors.

As will be shown in greater detail below, the defects in platelets and humoral and cell-mediated immunity have been well characterized by many different individuals. These defects and their pathophysiology however remain poorly understood and the cellular or metabolic basis for the disease remains unknown.

## GENETICS AND EPIDEMIOLOGY

Wiskott-Aldrich syndrome is fortunately a rare disorder with an estimated prevalence of less than one case per million individuals in the United States[14]. There is no known ethnic or socioeconomic predisposition for this syndrome. It has been reported in the medical literature of Japan, Italy, France, Poland, Germany, the Soviet Union, Hungary, Czechoslovakia, and the United States and probably exists elsewhere. It is however a disorder requiring a sophisticated medical care system for diagnosis and will probably not be reported at all in countries with a high infant mortality rate.

Aldrich et al[2] were the first to demonstrate the genetics of this syndrome by studying the transmission of the trait in a large family who had kept detailed family records for the preceeding four generations. Because of its sex-linked recessive nature the syndrome is passed from unaffected mother to affected son. Boys born to a carrier mother thus have a 50% chance of acquiring the disorder while daughters have a 50% chance of becoming carriers themselves.

Although this disorder is usually fatal early in life, a few males have survived to a reproductive age[15,16,17]. There are however no reports of them having fathered children. WAS can therefore be considered an X-linked lethal disorder wherein a certain number of WAS genes are removed from the gene pool each generation. To maintain the presence of this disorder in a population these removed genes must therefore be replaced

by new mutations. Haldane[18] calculated that with respect to an X-linked lethal gene one third of all affected males per generation would represent new mutations, assuming identical mutation rates in maternal and paternal germ cells and that the population was in equilibrium for this gene. These newly mutated males should therefore have normal homozygous mothers.

This hypothesis was tested by Francke et al[19] in families with Lesch-Nyhan disease, a well studied X-linked recessive and lethal disorder in which the carrier state can be readily detected[20,21]. They found that the incidence of new mutations in heterozygous mothers was close to that predicted but that the number of normal homozygous mothers with affected sons was significantly lower than predicted. They concluded from this that Haldane's theory might not be accurate, at least for Lesch-Nyhan disease.

Because of the inability to detect the carrier state in WAS we cannot test Haldane's hypothesis. However, two points should be inferred from this theory. The first is that adequate genetic counseling is very difficult without a method for detection of the carrier state. Currently we can tell the daughter who has two brothers with WAS, with fair certainty, that she has a 50% chance of carrying the disease. But what does one tell the daughter who has only one brother with WAS and an otherwise completely negative family history? Remember that a certain percent of affected males will be spontaneous mutations, but that a certain number of carrier mothers will be spontaneous mutations as well. In one case the daughter has a 0% chance of being a carrier and in the other a 50% chance. The need for a method to detect the carrier state is therefore quite obvious. Even if this

need were met however, many of the possible carriers still desire children. Carriers of course have a 25% chance of giving birth to an affected male with each pregnancy. A method for detecting WAS males in utero would thus be helpful for these families as well.

The second point to infer from Haldane's theory is that even if prenatal diagnosis and a method for detecting the carrier state were available, a certain number of carrier females and WAS males representing spontaneous mutations would still be born in previously unaffected families. This number is unknown but may be as high as 33% of all affected males currently being born if Haldane's hypothesis is correct. WAS will thus always be with us and an understanding of the disease and methods to effectively treat it still necessary.

In practice, the women of families with one or more WAS patient are divided into several groups: obligate carriers, probable carriers, possible carriers, and probable normals (see Table 1 for definitions). This division will in the future allow us to study the carrier state with greater precision for it is from this group that we feel the answers to WAS will be forthcoming.

## PATHOPHYSIOLOGY

The abnormalities of thrombocytopenia, eczema, and the multiple humoral and cellular immune defects seen in WAS are difficult to link together under a single pathogenetic mechanism. It must be remembered also that this is an inherited disorder and every cell in the body carries the WAS gene, although only a few seem to express it. Exactly which cell lines express the defect and which are responsible for the multiple defects of the disorder is still under debate.

The thrombocytopenia in affected males has been the best studied aspect of WAS. Its etiology is felt not to be secondary to the immune problems since it is almost always seen immediately at birth whereas the immune difficulties usually do not appear until several months later[11]. Severe hemorrhage is often reported at circumcision and melena or bloody diarrhea is often present within the first month of life[2,17,22]. Prothrombin and partial thromboplastin times are normal. Platelet counts, often immediately after birth, are in the range of 15,000 to 40,000 per $mm^3$ (normals for newborns are 125,000 to 250,000 per $mm^3$ and increase only slightly with age).

One of the first explanations for this thrombocytopenia was possible splenic sequestration since many of the affected children had slight to moderate splenomegaly on physical examination[3,5,23]. Many of the patients with chronic thrombocytopenia underwent splenectomy for this

reason. Most of these patients did indeed show a transient rise in platelet count after splenectomy but their counts invariably returned to their pre-surgery levels within a few weeks[24]. It was noted however that these splenectomized patients had a very high incidence of severe and often fatal pneumococcal infections within one to two months after surgery[3,24]. Because of this problem and the failure to correct the thrombocytopenia, splenectomy is now avoided unless other pressing medical needs dictate.

Another explanation for the thrombocytopenia had to be considered and the question of possible decreased platelet production was addressed. A review of bone marrow aspirate and biopsy results on these patients shows that most had normal to slightly increased numbers of megakaryocytes[8,17,22,24-27] although one group reported absent megakaryocytes in two out of their three patients[28]. Megakaryocyte morphology in these stained biopsies was usually reported as normal. However, Pearson et al[8] noted little active platelet formation despite the presence of nuclear hypersegmentation, karyorrhexis, and degenerate nuclei which are reportedly seen only when the megakaryocyte is actively shedding platelets. Baldini also felt the megakaryocytes in his patients were abnormal, expressing features of immaturity such as vacuolization and spongy cytoplasm[27]. Although no platelet production studies were done on these children it was generally felt that ineffective thrombopoesis was only partially responsible for their thrombocytopenia.

The major cause of the thrombocytopenia was felt by many (though

without good evidence) to be of an autoimmune nature similar perhaps to that responsible for idiopathic thrombocytopenic purpura (ITP)[29]. However, Pearson et al[8] showed that normal donor platelets infused into WAS boys had a normal survival time. Others, using $^{51}Cr$-labelled platelets from WAS children, showed markedly decreased platelet survival times when infused into both WAS males and normal volunteers[22,30]. This data implied that the thrombocytopenia was due to increased destruction secondary to some intrinsic platelet defect rather than merely decreased platelet production. The other implication of these experiments was that platelet transfusions could be given with confidence to WAS males when faced with surgery or other hemorrhagic events[8,31].

Studies on the morphology of the platelets from WAS males have been most interesting. It has been shown repeatedly by light microscopy, electron microscopy, and protein content assays that these platelets are on the average only 65% the size of normal platelets[22,25-27]. In addition, this abnormal platelet size has been confirmed by platelet-size distribution studies using particle-counting electronics (see figure 1)[32]. Some groups have reported that WAS platelets contain decreased numbers of alpha-granules, mitochondria, dense core bodies, and other internal structures despite their smaller size[22,27,30]. More recently however, others have reported normal numbers of intra-platelet organelles[26,32]. Gröttum[22] reported widespread phagocytosis of these abnormal platelets by marrow macrophages and concluded that the decreased platelet survival seen in these patients might be due to recognition and phagocytosis of the morphologically

abnormal circulating platelets by the reticuloendothelial system.

The question of abnormal platelet physiology in WAS has been the subject of great debate for the last ten years. It has been shown that platelets from many WAS males exhibit decreased nucleotide storage pools[22], defects in platelet adhesiveness in response to collagen[22,27], and failure to undergo the second phase of aggregation when stimulated with epinephrine, ADP, or collagen[22,25]. However, Hadden et al[33] felt that these in vitro results could be explained solely by the smaller size and lower number of platelets in the WAS patients. Others have reported that "if carefully concentrated" the WAS platelets displayed normal adhesiveness and aggregation[26].

For a solution to the small size and decreased number of platelets in the WAS males many investigators turned to the obligate carriers of this disorder since they have normal platelet size and numbers[32]. Although not considered at the time, this approach does not make sound theoretical sense for reasons that will be discussed at length later. Surprisingly however, many of the aggregation defects seen in the abnormal platelets of affected males have also been reported in their mothers despite the fact that the mothers were normal in every other respect and expressed none of the signs of the disorder seen in their affected sons[25,30,34]. These aggregation defects in the mothers appear to be variable however.

It has been recently proposed by Shapiro et al[34] that the defect of aggregation may be caused by the genetic absence of some physiological stimulator of the Krebs cycle. They felt that the platelets were still able

to undergo secondary aggregation but were merely more sensitive to inhibition of this aggregation than normal platelets. In addition, they have proposed a method for detecting the carrier state based on this principle. The details of these experiments will be discussed and criticized at length later in this thesis.

In summary, it is generally agreed that the bleeding problems in WAS males are secondary to decreased circulating platelet mass which itself results from the production of abnormally small platelets combined with shortened platelet survival time. Shortened lifespan has been attributed to intrinsic platelet defects or peripheral phagocytosis or both. The rate of platelet production is probably decreased but has not been proven. The basis for the production of these abnormal platelets is unknown. The physiological platelet defects have not been seen by all investigators and their existence and possible significance remain under debate.

Aside from the thrombocytopenia, the remaining abnormalities in WAS can be categorized as defects in cellular and humoral immunity. These defects however are difficult to tie together under one unifying hypothesis although many authors have tried.

Recurrent bacterial infections are the most serious problem in this disorder, accounting for 55% of all deaths[22]. WAS children are most vulnerable to infection with staphylococci, pneumococci, and a variety of gram-negative organisms despite the fact that they have a normal complement system and normal numbers of polymorphonuclear leucocytes with normal granulocyte function[5,9,10]. The only defect observed in this

system was by Spitler et al[35] who noted that granulocytes from four of nine WAS boys failed to adhere to nylon fiber columns. Bacterial killing and nitroblue tetrazolium tests were normal, however. This defect has not been reported elsewhere and its significance is unknown.

Total serum immunoglobulins are normal as are IgG and IgD fractions. Serum IgE levels have been noted to be elevated in some patients[36]. One of the classic findings in WAS however has been a persistently low IgM with slightly elevated IgA[5,9,10] although these levels are usually within two standard deviations of the normal mean. Another classical finding is the virtual absence of isohemagglutinins --naturally occurring IgM-class antibodies to the common blood group antigens-- in these patients[5,7,8]. Isohemagglutinin titers in blood group O WAS patients are in the range of 1:2 or less compared to titers of >1:16 seen in normal individuals. Immunoglobulin synthesis in response to protein antigens is often normal in affected boys whereas they almost always exhibit very poor antibody formation against polysaccharide antigens. Cooper et al[9] felt that the low IgM seen in many of these patients could be explained by their inability to make antibody against the polysaccharide antigens since much of the antipolysaccharide antibody in normal patients is of the IgM class. The slightly elevated IgA levels seen in some of these patients have not been explained.

Lymph node biopsies, at least early in life, usually show an abundance of germinal centers and plasma cells[9,13]. The thymus is present and undergoes normal morphological development[9]. The total lymphocyte

count in these children is normal as are the total numbers of circulating B and T-lymphocytes[11,35]. Despite the normal development of these cellular components however, most WAS patients are completely anergic. They show absolutely no delayed hypersensitivity response when skin-tested with ubiquitous antigens or with dinitrochlorobenzene (DNCB) sensitization[6,9,10,23]. Skin homograft rejection, another cell-mediated immune response, is often impaired in these children as well.

These results all imply a severely impaired cell-mediated immune response and may explain the increased susceptibility to viral and fungal infections often seen in these children[13]. Since cell-mediated mechanisms are also responsible for the cytotoxic T-lymphocytes postulated as playing a role in immune surveillance, a failure of these mechanisms may also explain the frequent development of lymphoreticular malignancies seen in these children.

Despite absence of cell-mediated immunity by *in vivo* skin testing, many authors have noted a normal or only slightly decreased response of lymphocytes to *in vitro* phytohemagglutinin (PHA) or mixed-lymphocyte stimulation[35,37,38]. These observations coupled with the evidence that the machinery for humoral and cell-mediated responses was present led Cooper *et al*[9] to suggest that the defect in WAS was in the *afferent* limb of immunity. They felt that the ability to process the antigen, the transfer of the immune message to the effector cell, or the ability of the effector cell to recognize the immune message was somehow defective.

Several authors have tried to explain the broad defects in immunity

as resulting from immunologic attrition as the affected child ages. Cooper et al[6,9] suggested that the variable results of immune function studies were dependent on the age at which the patient was tested. Younger children, for example, have normal lymph nodes and respond fairly well to a number of protein antigens. In contrast, older patients often display marked degeneration of parafollicular (T-lymphocyte) and germinal center (B-lymphocyte) areas at lymph node biopsy and often show poor response to protein antigens. Steihm and McIntosh[28] and others[7] showed that the classic immunoglobulin picture of low IgM and elevated IgA evolves with time over the first year or so of life. Cooper et al[9] then suggested that the attrition may be related to an inability to process polysaccharide antigens and that the efferent limb of immunity atrophied secondary to lack of stimulation.

Blaese et al[10] also felt that this defect might lie in the failure to process antigens and they postulated a possible defect of monocyte/macrophages. These cells are known to play a very important role in presenting antigens to certain lymphocyte subpopulations in the afferent limb of immunity[39] and their failure to do this might certainly produce defects in both humoral and cellular immunity. Kuramoto et al[25] suggested that the alpha-granules in the macrophages as well as the platelets might be metabolically defective but this has not been proven. Since normal macrophages play a large role in phagocytizing opsonized antigens the ability of WAS macrophages to do this was investigated. Spitler et al[35] examined the IgG receptors on monocyte/macrophages from affected children and found

slightly diminished receptors in some but not all of their WAS patients. The presence of this finding did not correlate with the severity of the disease and its significance is unknown. Others have reported normal monocyte IgG receptors in their WAS patients[17]. In addition, Blaese et al[40] incubated normal macrophages with lymphocytes from WAS patients and were unable to show improved lymphocyte response to in vitro antigen stimulation. They concluded that the basic defect in WAS did not involve monocyte/macrophage function.

The eczema seen in all of these patients is the most difficult manifestation of the disorder to explain. This partly stems from the fact that we still know very little about the pathophysiology of eczema even in normal individuals. Many feel that the eczema seen in normal patients is a type of cell-mediated immune reaction but this explanation is doubtful in WAS patients since they appear to have absent or severely diminished cell-mediated immunity. Many of these affected boys have skin-sensitizing antibodies to common allergens and a few have developed food allergies[9], responses which are IgE-mediated in normal patients. WAS males often have elevated IgE levels and eosinophilia but it is unknown whether these are a cause of their immediate hypersensitivity symptoms or merely a secondary result of other immune deficiencies. The eczema in WAS may well be mediated by the elevated IgE or eosinophilia or may be secondary to the major humoral and cell-mediated defects. However, no one has yet to propose a pathophysiological mechanism.

Perhaps more light can be thrown on the confusing picture of these

multiple immune defects by considering the results of treatment of this disorder. Several different modalities have been tried, but can be classified under two basic categories: immune or hematologic support, and actual attempts to correct the cause of the immune defects. Support in the form of immune globulin injections or plasma, erythrocyte, and platelet transfusions have prolonged life and ameliorated some acute infectious phases but on the whole have not improved the miserable immune functions in these patients[7].

Better results have been shown with attempts to correct the unknown cause of the immune defects. The two most promising methods in the last few years have been bone marrow transplants and treatment with transfer factor. Transfer factor treatment, since it is simple and has few risks, might show great potential for therapy of WAS. Transfer factor is a small dialysable molecule prepared from sensitized leucocytes of normal individuals which is able to transfer specific immunity to deficient patients by an unknown mechanism. It has shown great potential in the treatment of chronic mucocutaneous candidiasis[41].

Transfer factor therapy in WAS has been reported in some cases to clear or improve the eczema, reduce infections, and decrease the frequency of bleeding episodes, although not correcting the thrombocytopenia[17,42]. These changes however are inconsistently seen and the effect of transfer factor is difficult to judge because the course of WAS is so highly variable. Most recently, a controlled double blind clinical trial of transfer factor in the treatment of WAS has shown no <u>in vivo</u> or <u>in vitro</u> improvement of

cellular reactivity and no improvement in clinical condition of four WAS patients[43].

The best results in treatment of this disease have been obtained with complete autologous bone marrow transplantation which, in two cases, has restored these children to completely normal immune and hematologic function[12]. It must be realized however that bone marrow transplantation is suitable for very few WAS patients. As with transplantation of other organs, the major difficulty is in finding an HLA-compatible donor. Other major problems include the dangers of potent immunosuppressive therapy, infection, and graft-versus-host disease. The remarkable results obtained in these two cases however can shed some light on the possible location of defects in WAS.

The first bit of information is that WAS can be totally cured by complete stem cell replacement. In one of the cases however, the first bone marrow transplant failed. The only marrow cell line that "took" in that first graft was the T-lymphocyte line. This resulted in complete clearing of the child's eczema. His anti-A isohemagglutinin titer, which had been 1:4 prior to transplantation, rose to 1:64 and his concentration of antibodies to polyribose phosphate (a polysaccharide antigen) rose as well. Over the next nine months his T-lymphocyte karyotype gradually returned from female (donor) to male (host). Despite the return of his own T-lymphocytes, the child's eczema did not recur. At the end of nine months the patient's hemopoetic and lymphoid cell lines were entirely of recipient karyotype with no evidence of persistence of any donor cells. Anti-A and B isohemagglutinin

titers had dropped to 1:2 and the anti-polyribose phosphate antibody concentration had dropped as well.

This T-lymphocyte replacement and gradual return along with the marked improvement in immune response and its gradual return to the pre-transplantation levels is compelling evidence that the defect in the immune system of these WAS children lies within the T-lymphocyte. The possibility that another cell line such as the monocyte "took" along with the T-lymphocytes, was responsible for the immune changes, and then returned to host karyotype along with the lymphocytes cannot be ruled out, of course. The authors make no mention of having looked at any hematogenous cell populations other than lymphocytes, granulocytes, and platelets.

The inherited genetic nature of the disease implies that every nucleated cell in the body of affected males carries the gene. The marrow transplant results however imply that the WAS defect is expressed only in cells of bone marrow origin. Exactly which of the stem cell progeny actually express the WAS gene remains unknown. We do know that platelets and, by inference, megakaryocytes are affected. Some authors have suggested that the immune problems are a result of defective monocyte/macrophages[9,10] or a B-lymphocyte subline[11], although the B-lymphocyte theory was disproven just prior to publication. The marrow transplantation work implies that the defect may be solely in the T-lymphocyte. One group has even reported a defect, perhaps incidental, in the granulocyte. The only hematogenous cell line that no one has implicated is the erythroid series.

The primary defect in WAS might be expressed in a single hematogenous cell line which, by its defective interaction with other normal cell lines, may then lead to a chain reaction culminating in the observed broad immune defects of the syndrome. On the other hand, all of the cell lines or perhaps even just a subgroup may be expressing the gene. Our understanding of the complex interactions of these different lines in normal individuals is still so rudimentary that an answer to the problem in WAS still eludes us.

Attempts to locate the expression of the WAS defect to particular cell lines are thus a valid approach to understanding not only this disorder but the network of cellular interactions in the normal system as well. This approach may also point us in the direction of specific immune therapy for WAS children in the future.

## THE CARRIER STATE

One possible way to locate the particular cell lines responsible for WAS might be to examine the expression of this disorder in the carrier mothers since these women in theory should be heterozygous mosaics for the trait. In the early work on this disorder many attempts were made to identify the carrier state but it was noted time and time again that the obligate carrier mothers were completely normal. They had no problems with infections, no eczema, and no thrombocytopenia. It was shown that the concentrations of their serum immunoglobulins were normal[28], as were their isohemagglutinin titers. They had normal lymphocyte numbers and function, and their cell-mediated immunity responses to delayed hypersensitivity skin testing and in vitro PHA stimulation were normal as well[35].

Hematocrits and platelet counts in carrier mothers are normal, as are their platelet survival times. In addition, unlike the small platelets seen in the affected males the platelets from obligate carriers have a normal size distribution when compared to normals (see figure 1)[27,32]. Their platelet protein content --a rough measure of volume-- is normal and no abnormalities of platelet organelles have been seen under the electron microscope[27].

In contrast to this boring normality however, platelet physiology appears to be abnormal in many of these carrier mothers. Baldini[27] noted that although platelet adhesiveness in response to collagen was

TABLE 1. Classification of WAS carriers: Definitions

| | |
|---|---|
| 1. Obligate carriers: | Women with two or more WAS sons; or women with one WAS son and a positive family history for WAS. |
| 2. Probable carriers: | Women with only one WAS son and an otherwise negative family history. Also appearing in this group are daughters of obligate carriers who have no WAS children but with WAS appearing in the families of their female descendants. |
| 3. Possible carriers: | Women who are daughters of an obligate carrier, but who have not had any male children, and have no WAS children in the families of their female descendants. |
| 4. Probable normals: | Women with a positive family history for WAS but who have had one or more normal sons but no WAS sons, and in whose female descendants' families there are no WAS boys. |

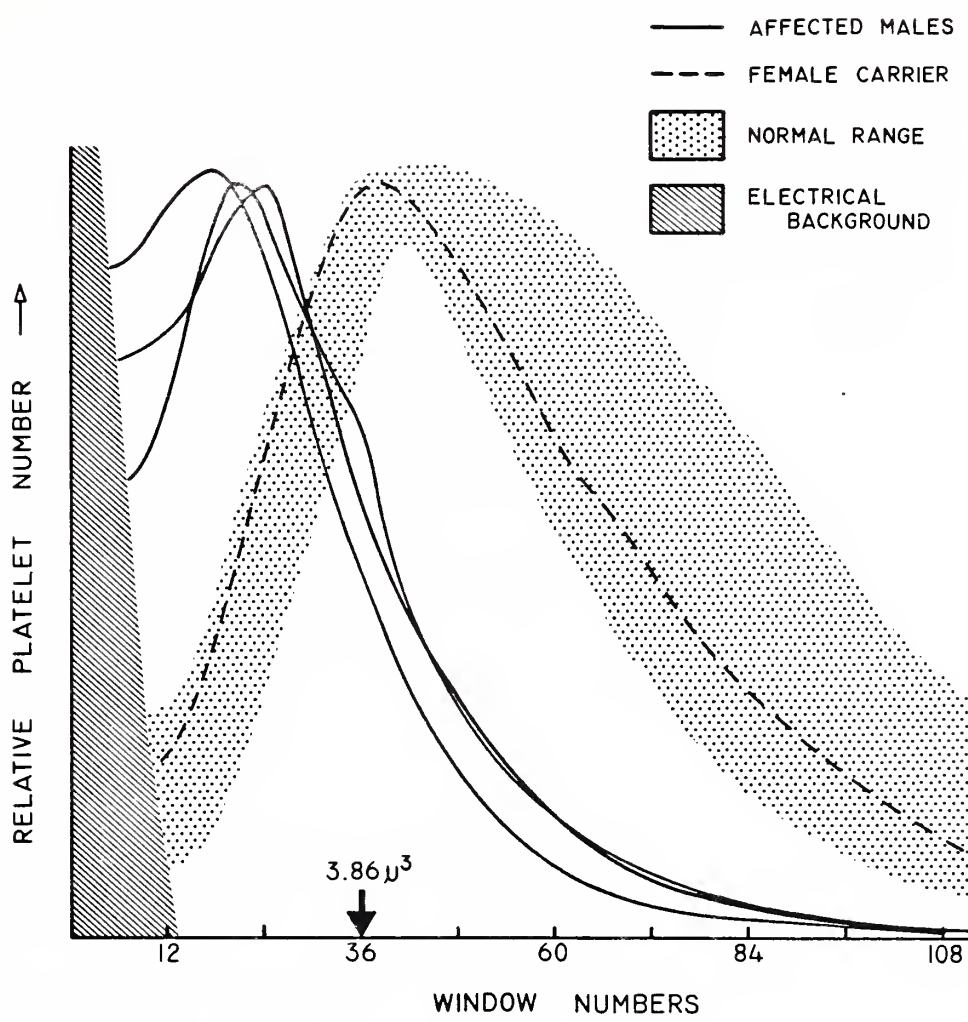


FIGURE 1. Platelet size distribution in Wiskott-Aldrich syndrome.

With the use of an Electrozone Celloscope (Particle Data Corp., Elmhurst Illinois), which divides the cell population into 128 windows, Murphy et al[32] showed that three affected brothers had abnormally small platelets, whereas their mother's platelets fell within the normal range. The standard, a polyvinyltoluene particle with a volume of 3.86 $\mu^3$ peaks in the window marked by the arrow. It should be noted that the curve for the mother's platelets falls completely within the normal range and does not display a peak or shoulder in the windows in which the smaller platelets of her affected sons are found. This indicates that the mother does not have a dual population of normal and abnormal sized platelets (see text for further explanation).

This graph is a representation of the work done by Murphy et al[32]: N. Engl. J. Med. 286(10):499, 1972 and is used here as an illustration only.

normal in the two mothers he studied, the second phase of platelet aggregation in response to epinephrine, ADP, and collagen were almost completely lacking in one mother (see figure 2). The other mother was missing only the secondary aggregation in response to epinephrine, the responses to ADP and collagen being normal. As mentioned previously, these platelet defects are seen in the affected males as well.

Baldini also noted that when platelets were incubated with latex beads normal individuals showed a ten to twelve-fold increase in $^{14}CO_2$ production from $^{14}C$-glucose. This response was "defective" in the WAS mother and no reaction was seen at all in the affected sons[30]. Kuramoto et al[25] were able to repeat this work on a different mother. They showed that, like her two WAS sons, she was missing the second phase of platelet aggregation in response to epinephrine. In addition, the production of $^{14}CO_2$ and $^{14}C$-lactate from $^{14}C$-glucose by the mother's platelets when stimulated with epinephrine was intermediate between the levels seen in her sons and a normal control.

However, in a second mother that Baldini studied, the aggregation defect was found only with epinephrine and could not be demonstrated in every determination[27]. He also mentioned that another group observed a defect of platelet aggregation with epinephrine that was clear-cut but inconstant in three WAS mothers.

Most recently, Shapiro et al[34] studied six different families containing eight obligate and two probable WAS carriers. They found that the secondary aggregation in response to stimulation by collagen, thrombin,

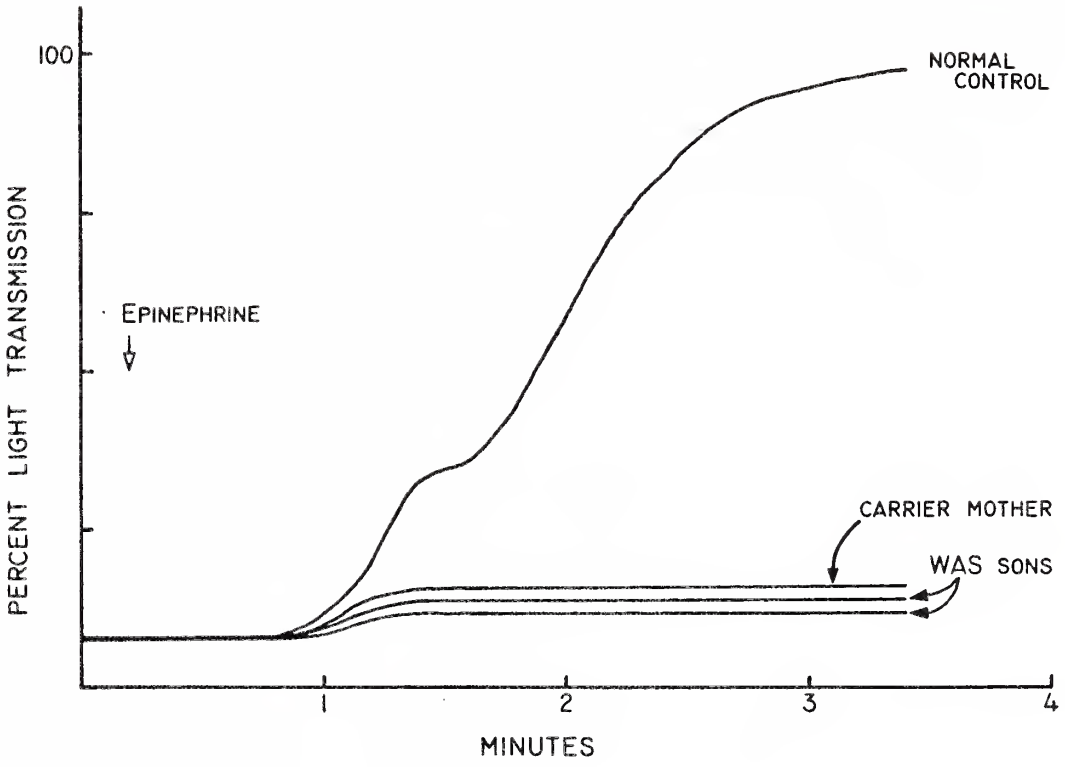


FIGURE 2. Schematic representation of results of platelet aggregation using epinephrine with the platelets of two affected WAS children and their carrier mother.

The control curve was obtained with platelets from the normal sibling. Platelets were collected in citrated blood and adjusted to 500 000 per µl in autologous plasma. Aggregation was studied by the use of an aggregometer recording the changes of optical density.

These results were obtained by Kuramoto et al[25], N. Engl. J. Med. 282(9):475, 1970 and their graph is shown here to illustrate the aggregation defect reportedly found in carriers and affected males by several different authors (see text).

ADP, and epinephrine was completely normal. In order to see if they could elicit an aggregation defect they undertook an experiment to selectively block one or the other of the two metabolic pathways that generate ATP required for secondary aggregation. By selectively blocking the glycolysis pathway they were able to elicit the aggregation response only in normal platelets. The platelets from the WAS carriers failed to aggregate exactly as described by Kuramoto et al[25] and Baldini[27]. If Shapiro et al blocked only oxidative phosphorylation both normal and WAS carrier platelets underwent normal aggregation. If both pathways were blocked simultaneously, both groups of platelets failed to aggregate. Because WAS carrier platelets were sensitive to blockage of glycolysis, Shapiro et al concluded that these platelets already contained an intrinsic blockage of oxidative phosphorylation, and that the molecular defect in WAS was possibly the absence of some molecular stimulator of oxidative phosphorylation or the Krebs cycle.

This is an attractive hypothesis. However, it now appears that this elicitable aggregation defect is often variable and can be seen, for example, in normal men and normal pregnant women[14]. In addition, our understanding of platelet physiology responsible for secondary aggregation responses is in its infancy. Several authors have recently observed that certain lipophilic extracts of onions[44], or even a single meal containing onions[45], could completely or partially block the second phase of platelet aggregation in normal individuals. These results certainly cloud the validity of the findings seen in the platelets of WAS carriers.

Let us stop for a minute and consider some of the genetic aspects

of WAS and the carrier state. It is an accepted fact that WAS is an X-linked recessive disorder. As in other X-linked recessive diseases, it is presumed that males express the trait because they express only one X-chromosome, and that heterozygous (carrier) females do not express the trait because they have two X-chromosomes, the normal allele on one "making up" for the defective allele on the other. This pattern is seen in several X-linked recessive disorders such as color blindness, Lesch-Nyhan disease, hemophilias A and B, and Duchenne's muscular dystrophy[46].

It is widely accepted however that all females are mosaics regarding X-chromosome activity. This hypothesis of mosaicism was first postulated in 1961 by Mary Lyon[47] in an attempt to explain X-chromosome dosage compensation in females. Although called the Lyon Hypothesis for historical reasons, it is now regarded as proven fact. It has been shown that one of the two X-chromosomes in each cell becomes genetically inactive at a very early stage of embryonic development. Which of the two X-chromosomes becomes inactivated is a random event, but once that inactivation takes place that same chromosome remains inactivated in all the progeny of that original cell. Each female is therefore a mosaic with roughly half of her cells expressing the paternal X-chromosome and the other half expressing the maternal X-chromosome. If a female is a heterozygous carrier for a mutant allele on the X-chromosome, on the average half of the cells in each of her tissues will be normal and the other half will express the mutant allele.

For example, even though the carriers of hemophilia A or B manifest

no bleeding problems, they have been shown to have anti-hemophilic factor levels intermediate between normal levels and the absent or greatly diminished levels of their affected sons[46]. Lesch-Nyhan disease is similar in that affected males lack the enzyme hypoxanthine phosphoribosyl transferase (HPRT). The HPRT levels in most tissues of the carrier mothers are intermediate between affected males and normal patients. In addition, cloning studies have shown that, on the average, half the individual cells in the carrier mothers express full HPRT activity and the other half individually express no activity at all[20].

In these cases, the normal cells in the carrier mothers produce enough anti-hemophilic factor or HPRT to make up for the deficient cells and prevent the mothers from manifesting any form of the disease. Occasionally, however, a woman heterozygous for an X-linked recessive trait will, by the ill luck of the draw, express a greater percentage of mutant allele than normal allele-bearing cells and thus may express a mild to severe form of the disease depending on the percentage of cells expressing the mutant allele. This happens rarely but has been seen, for example, in hemophilia and glucose-6-phosphate dehydrogenase (G6PD) deficiency[48]. In these cases the percent of cells expressing the normal allele in each tissue is so small that the product or effect they produce cannot completely make up for the deficiency expressed by the majority of cells. Under these circumstances then, a heterozygous woman may express all or part of an X-linked recessive disorder.

Let now return to the Wiskott-Aldrich syndrome. If we accept the

Lyon Hypothesis as fact, then the obligate carriers of WAS should be mosaics for the disorder. In other words, roughly one half of the cells in each tissue should carry the mutant WAS allele and the other half should carry the normal allele. We might thus expect to see immunity and platelet defects in the obligate carriers that are intermediate between normal and the severe defects seen in their hemizygous affected sons. We know however that the mothers are completely normal except for some proposed minor defects in platelet physiology.

One might argue that the immune defects are not seen in the mothers because their normal cells are able to compensate in some way for the defects expressed by the abnormal WAS cells. For example the normal cells might produce a stimulating factor or provide a necessary cellular function not found in the abnormal cells. The carrier mothers could then have a qualitatively normal immune system despite a dual population of affected and normal cells.

An alternative and equally feasible explanation for the normal immunity in the carrier mothers might be that the abnormal cells expressing the WAS gene have been selected against. One method by which this could happen is non-random inactivation of only the chromosome bearing the WAS allele. However, non-random X-inactivation has been seen in humans only in the case of those females having one normal chromosome and one structurally abnormal chromosome[49]. The X-linked genes of these individuals are thus expressed as in a male.

A second method by which the WAS cells could be selected against

is competition. If the WAS cells in each tissue were in some way less competitive for survival they might be replaced in time by the normal cells simply because the normal cells were more viable. If, as has been suggested, the cells in which the WAS gene is expressed have a defect in energy production via oxidative phosphorylation, then this idea is entirely plausible.

Exactly which cell lines in the carrier mother could be selected against in this fashion is open to conjecture. All the cells in the affected boys carry the WAS gene, but the defect appears to be expressed only in cells of hematogenous origin. We might find in the carrier mothers that all the cells that carry the WAS gene have been replaced, but this is doubtful. A more likely finding might be that only the cells that express the WAS defect are selected against. If this were indeed the case, and if the WAS gene was initially expressed in the same cell line in mother and son prior to selection, then it might be possible to determine the defective cell line in the WAS boys by studying the selection patterns in their mothers.

Suppose for example that the WAS defect is expressed only in the monocyte/macrophage. If our selection theory is true then we would expect that the defective monocyte/macrophage in the carrier mother had been selected against at some point in development and the mother would then have only one population of monocyte/macrophages: those expressing only the normal allele. If we did indeed find this in obligate carriers then we might infer that the monocyte/macrophage is the cell responsible for the expression of the disorder in the affected males. This of course rests on

the assumption that selection occurs only against cells that express the WAS defect, and that the defect is expressed in the same cell lines in mother and son.

Let us now consider the case of the platelets in the carrier mothers. If the obligate carriers are mosaics for WAS and no selection has taken place, then these women should have two populations of megakaryocytes in the bone marrow. One would therefore expect to find two populations of platelets in the peripheral blood. The population of platelets derived from normal megakaryocytes should have normal morphology and physiology, and the population derived from WAS megakaryocytes might be expected to resemble identically the abnormal platelets seen in the affected males.

One must remember however that the platelets from WAS males have a reduced survival time, even when infused into normal persons[22]. If we assume that the thrombocytopenia in affected males is due solely to increased destruction, that their rate of platelet production is the same as in normals, and that the two platelet populations in obligate carriers follow these criteria then we would expect a reduced survival time in the carrier platelets derived from the WAS megakaryocytes. Thus even if the carrier had equal populations of WAS and normal megakaryocytes one would expect that most of the platelets in the peripheral blood would be of the normal type. The average platelet count in affected males is around 25,000 per $mm^3$ whereas the average platelet count in normal adults is, say 250,000 per $mm^3$. If the same processes giving rise to these counts in affected boys and normals are going on simultaneously in the carrier mothers then

one might expect that roughly ten percent of their platelets would be of the abnormal type while the remaining ninety percent would be of the normal type.

However, the experimental evidence accumulated to date does not support the idea of two separate populations. No one has found a population of abnormally sized platelets in the carrier mothers. Perhaps the best evidence for this is the platelet size distribution study done by Murphy et al[32] (see figure 1). If two populations of different sized platelets existed in the carrier that this group studied, one would expect the size distribution curve to exhibit two peaks; one smaller peak corresponding to the abnormal platelet pool and a much larger peak corresponding to the normal platelet pool. Their technique was sensitive enough that it should have been able to detect the presence of abnormally sized platelets in the carrier even if that pool made up only ten percent of the total platelet count. Their results, however, show only one peak; that corresponding to the pool of normal control platelets.

There are two ways to explain this result. The first possibility is that there is indeed a pool of abnormally small WAS platelets but it comprises much less than ten percent of the total platelet count and thus cannot be differentiated from background on our sizing machine. The second possibility is that there are indeed no small platelets present. For practical purposes however, the first possibility can be considered a subcategory of the second possibility, as will be demonstrated below.

If we assume from this information that there are no small platelets

present in the peripheral circulation of the obligate carriers then there are three possible explanations. Let us assume first that the carrier mother is a mosaic for WAS and had two equal populations of megakaryocytes. The first and least likely explanation for the apparent absence of abnormal platelets is that they are indeed produced but are destroyed so rapidly in the periphery that we cannot detect them. If we could detect the presence of the WAS allele in a cell population we would then expect to see a heterozygous megakaryocyte population but only a hemizygous normal platelet population.

The second explanation for the absence of small platelets, still assuming a mosaic population of megakaryocytes, is that the abnormal megakaryocytes are now producing normal size platelets, perhaps in response to some unknown factor that is missing in the affected males but is now supplied by the normal cells in the carrier mother. If this were the case then we would expect to see a genetically heterozygous population of platelets as well as megakaryocytes.

The third and most attractive explanation however, is that even though the carrier mother is a mosaic for WAS, she has a non-mosaic population of megakaryocytes. The megakaryocytes carrying the WAS allele might have undergone non-random X-inactivation or they might have been selected against and overgrown by her normal megakaryocytes. All of her platelets, since they were derived from normal megakaryocytes, should thus have a normal size distribution and normal physiology. In this case we would expect to see a genetically hemizygous normal population of

both platelets and megakaryocytes.

If this latter theory is true, how then can one explain the work on carrier platelets which is reported to show defects in secondary aggregation and putative defects in oxidative phosphorylation? This work implies that all or most of the carrier platelets are physiologically abnormal. If the carrier mothers are indeed mosaics and no selection has taken place then at the very least a large number of their platelets should be completely normal. If, in addition to the mosaicism, selection against the WAS megakaryocytes has taken place then all of their platelets should be normal and express none of the defects reportedly seen. Yet the physiological defects do seem to be present, at least according to some authors.

Three possible explanations that consider both the size data and the physiological data as well as the requirements of the Lyon Hypothesis come to mind. The first explanation assumes that the two mosaic populations of megakaryocytes are present and that the normal cells exert some unknown positive influence on the WAS megakaryocytes, enabling them to produce normal sized platelets. Cells carrying the WAS gene then exert some negative influence upon the normal sized platelets causing them to exhibit abnormal physiological responses. Shapiro et al[34] suggested that the platelets from WAS carriers lacked an enzyme critical to ATP generation via the Krebs cycle and oxidative phosphorylation. Since our mini-theory assumes that most of the platelets in the carriers are of genetically normal origin they should not be lacking any necessary enzyme, but perhaps the putative physiological defect can be explained by a soluble factor origin-

ating from the WAS cells that inhibits one of the necessary Krebs cycle enzymes.

The second explanation is identical to the first except that we now make the assumption that selection against the WAS megakaryocytes has taken place and all the platelets are now of genetically normal origin. We now no longer need the postulated positive influence and can reconcile the physiologic and anatomic data with the proposed existence of only a single negative influence causing abnormal physiological responses in genetically normal platelets.

The third explanation that considers the Lyon Hypothesis, the size data, and the physiologic data is that the size data, or the physiological data, or both could be wrong. The size data, however, appears to be believable, since Murphy et al[32] (see also figure 1) could readily detect the smaller sized platelets of the WAS boys. On the other hand, the reliability of the physiological data may be seriously questioned, as pointed out previously. If this data is false and the physiological defects do not exist then the remaining observations tend to support a clonal selection theory, at least for the megakaryocytes.

At any rate, it should be obvious that a knowledge of the interactions of the normal and abnormal cells in the mosaic carriers of WAS might be of great help in answering questions about the cellular origin of the defect in affected males as well as the question of why the heterozygote carriers appear unaffected immunologically. An understanding of these interactions might also solve much of the controversy over the existence of abnormal

platelet physiology in the carriers and perhaps point us in the direction of a method for detecting the presence of the carrier state.

How then can we approach this problem? The ideal method would be to analyze each different tissue or cell line in the carrier for the presence or absence of the WAS allele. We are as yet unable to do this. If, however, we could detect the presence of a product of a different gene on the same X-chromosome, we could then use the product of this other gene as a "genetic marker" for the presence or absence of the WAS gene in a single cell, a clone, a cell line, or a heterogeneous tissue. This "genetic marker" technique has been used frequently to study several other X-linked recessive diseases, most notably Lesch-Nyhan disease, hemophilias A and B, and Hunter's syndrome. The technique has been used most extensively to unravel the behavior of the normal X-chromosome in females and to gather data in support of the Lyon Hypothesis.

The X-linked genetic marker most commonly used for these experiments is G6PD, which has two common isoenzyme variants that are easily detected. The ideal advantages of this marker will be discussed in a moment. If, however, we could follow the distribution of the two G6PD isoenzymes in the cell populations of mothers who were heterozygous for both WAS and G6PD, it could give us an excellent understanding of Lyonization and cell selection processes in these women, as well as answer many of the other questions we have outlined previously. Indeed, this is exactly what we have done.

## GLUCOSE-6-PHOSPHATE DEHYDROGENASE AS A GENETIC MARKER

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme found in all cells which participates in the metabolism of glucose via two major pathways, the hexose monophosphate shunt (HMP) and the Embden-Meyerhof pathway of anaerobic glycolysis. The HMP supplies the cell with NADPH, with 5-carbon sugars required for the synthesis of nucleotides, and maintains glutathione in the reduced state. Anaerobic glycolysis is most important in erythrocyte metabolism where it supplies ATP to power the ion pumps and to phosphorylate membrane proteins. It also supplies the cell with NADH to maintain hemoglobin in the reduced form[48].

Deficiency of this enzyme was discovered as a result of studies on the hemolytic anemia induced in some individuals by the antimalarial drug primaquine, a strong oxidant. The history of this study makes a fascinating story and has been covered well by Beutler[48].

In the course of study of G6PD deficiency over eighty different forms of the enzyme have been discovered, some with normal activity and some with reduced activity. Most of these isoenzymes are quite rare. The most common deficient enzyme has been classified as $A^-$, the A referring to its electrophoretic mobility and the ($^-$) referring to its decreased activity in the red blood cells (RBCs). When the $A^-$ enzyme is first produced within the cell its activity is very close to normal but the lifespan of the individual enzyme molecule is considerably shortened. Thus, reticulocytes from

an individual who is G6PD-$A^-$ express normal enzyme activity at first, but rapidly lose activity as the cell ages and the enzyme degrades. These older cells with decreased enzyme levels are then more sensitive to hemolysis in the presence of oxidizing drugs. However, nucleated cells from an individual who is G6PD-$A^-$ have normal levels and activities of this enzyme regardless of cell age since they contain a nucleus which continues to produce active enzyme and thus maintains a normal activity within the cell[50,51].

The most common normal G6PD isoenzyme is classified as G6PD-B, the B referring to its electrophoretic mobility. The most common normal variant is G6PD-A which, unlike the $A^-$ variant, does not degrade with time and thus has normal activity. The A variants migrate faster than the B variant on electrophoresis. The reason for this difference in electrophoretic mobility is the presence of a single amino acid substitution; an asparagine in the B variant is substituted for an aspartic acid in the A variant[52,53].

The genotype for G6PD is denoted as $Gd^x$, the (x) denoting the specific isoenzyme type. For example, a woman heterozygous for G6PD types A and B would have a genotype designated by $Gd^A/Gd^B$. Since the gene controlling the G6PD isoenzyme type is located on the X-chromosome, males are hemizygous, i.e., they possess only one G6PD gene since they have only one X-chromosome. Since females have two X-chromosomes they possess two G6PD genes and can be either homozygous or heterozygous for this locus.

As mentioned previously, the B isoenzyme is the most prevalent type. The incidence of the A, $A^-$, and other variants varies widely according to

ethnic group. For example, the A and $A^-$ variants are most common in groups of black African ancestry[54,55]. The gene frequencies of the most common isoenzyme types in the United States are listed in Table 2. As can be seen from this table, the combined frequency of $Gd^A/Gd^B$ and $Gd^{A^-}/Gd^B$ heterozygotes among American black women is 0.42. In other words, 42% of American black females are G6PD heterozygotes.

The G6PD enzyme system is an ideal one for us to use as a genetic marker. The enzyme is the single protein product of a single gene[53]. It can be isolated as a pure product from hemizygous males, it is found in all cells of the body, it is fairly easily identifiable by electrophoresis on cellulose acetate or starch gels, and it has been widely used with good success in the past. One of the special advantages of the G6PD isoenzyme system as a genetic marker is that neither the A, $A^-$, nor B isoenzyme is detrimental to the cell. In other words, a cell is at no selective advantage or disadvantage if it expresses one of these isoenzyme types since each type has the same activity.

In addition, much work has already been done on the distribution of G6PD isoenzyme types in cell populations of normal individuals. It has been shown time and time again that tissues from a G6PD heterozygote can be separated into two clones, each expressing only one isozyme[49]. Both Migeon and Kennedy[56] and Fialkow[57] have shown that inactivation of G6PD genes (and by inference, the whole X-chromosome) takes place early in embryonic development, the latter author estimating that this process might occur as early as the 16-cell embryoblast stage. Gartler et al[58]

TABLE 2

I. Polymorphous glucose-6-phosphate dehydrogenase gene frequencies in the United States.

| population group | G6PD isoenzyme types B | A | $A^-$ | other |
|---|---|---|---|---|
| blacks | .70 | .18 | .12 | sp |
| non-blacks | 1.00 | | sp | sp |
| Eskimos | 1.00 | | | |

(sp = sporadic reports)

II. Glucose-6-phosphate dehydrogenase genotype frequencies in American blacks, assuming Hardy-Weinberg equilibrium.

| | G6PD genotypes BB | AB | $A^-B$ | (AA, $AA^-$, $A^-A^-$) |
|---|---|---|---|---|
| frequency | .49 | .252 | .168 | .09 |
| combined frequency | .49 | .42 | | .09 |

Data for both I and II are derived from Betke et al[55]: World Health Org. Techn. Rep. Ser. #366, p 4 (1967).

studied X-inactivation by simultaneously following the isoenzyme patterns of two different enzymes widely separated on the X-chromosome. They found a woman who was doubly heterozygous for both HPRT deficiency and G6PD, and then studied the recombination patterns of these four markers. They found that single cell clones (all the cells of which should be expressing the same X-chromosome) always contained the same two isoenzyme pairs. For example, each clone contained either G6PD-B and (+)HPRT activity, or G6PD-A and (-)HPRT activity, but never a combination of the two. The authors inferred from this pattern that the entire X-chromosome within a given cell was inactivated, and that no crossovers occurred between activated and inactivated X-chromosomes within the cell.

Others have shown that the ratio of G6PD isoenzymes within a single normal heterozygote appears to be remarkably constant from tissue to tissue, and cell line to cell line. This has been observed in erythrocytes, granulocytes, lymphocytes, skin fibroblasts, and skeletal muscle by Fialkow[57]. This constancy has been extended to other cell types as well, most notably platelets[59], T-lymphocytes[60], and monocyte/macrophages[61]. These observations have great bearing on the experiments we have proposed, as will be discussed later.

Several authors have applied this knowledge of G6PD isoenzyme distribution to the study of the clonal origin of various tumors and other diseases[62,63]. By observing that certain cell groups from G6PD heterozygotes with these various diseases contained only one isoenzyme type rather than the mixture seen in normals, the clonal origins of chronic lympho-

cytic leukemia[60], chronic myelocytic leukemia[61,64], agnogenic myeloid metaplasia[65], polycythemia vera[59], atherosclerotic plaques[66], as well as a host of solid tumors[67] have been determined.

Polymorphous G6PD is thus an excellent marker for studying the X-chromosome. It is the most common X-linked genetic variant known, the isoenzymes can be easily differentiated chemically, and the presence of one isoenzyme over the other is of no selective advantage to the cell. Most important, this enzyme has been very well studied in normal individuals, and the ratio of isoenzymes is remarkably constant from tissue to tissue. All of these reasons make it a very attractive marker to use in attempting to understand the genetics of the WAS carrier state.

## HYPOTHESIS AND EXPERIMENTAL PROTOCOL

Although genetic markers have been widely used to study a number of X-linked diseases, to our knowledge no one has attempted to apply these techniques to the study of X-linked immunologic disorders. WAS is one such immunologic disorder in which the pathophysiological concepts remain unclear despite intensive immunological study. Perhaps a new or at least different approach to this disorder is warranted. As we have outlined in detail in the previous sections, there are many questions which might be answered by a study of the expression of the WAS gene in cell lines of the carriers. Some of these questions are outlined below and many are, of necessity, interrelated.

1. Does selection take place against the WAS cells in the carrier mothers, and if so, in which cell lines?

2. Why do WAS carriers appear not to express an intermediate form of the disorder?

3. How can the platelet size information of Murphy et al[32] be reconciled with the Lyon Hypothesis of mosaicism in the mothers?

4. How can the platelet physiology defects reported by Baldini[27], Kuramoto et al[25], and Shapiro et al[34] be reconciled with the Lyon Hypothesis?

5. Can the putative defective cell line(s) responsible for the disorder in affected males be determined by studying the carrier mothers?

6. Can a method for detecting the carrier state be found?

The solutions to the latter five questions hinge, to a great extent, on the answer to the first question: does selection take place, and if so, where?

This is the question we tried to answer from the outset. Once we knew the answer to this we could reevaluate the remaining questions and formulate new hypotheses.

Our approach to this first question, although simple in concept, proved to be slightly more difficult that we had planned; an occurrence not unusual in the pursuit of science. Briefly, we planned to locate one or more women doubly heterozygous for G6PD and WAS. Since the expression of the defect in the affected males appears to be limited to the bone marrow and its hematogenous progeny we felt we should begin our study with these elements. We then took whole blood from the carriers and separated it into its separate components: platelets, erythrocytes, granulocytes, monocyte/macrophages, T-lymphocytes, and B-lymphocytes. We then analyzed each cell group for G6PD phenotype to see if any cell group expressed a hemizygous phenotype rather than the heterozygous pattern seen in all of several normal controls. Our methods, results, and conclusions are detailed below.

## MATERIALS AND METHODS

I. Location of families In order to initiate our study of WAS carriers using linked genetic markers we first had to find an obligate or probable WAS carrier who was also heterozygous for G6PD. One individual who met these requirements was located through review of the literature[12] but she was residing in Lebanon and not immediately available for study. The remainder of our search was confined to black women since this is the only group in which polymorphous G6PD occurs with any frequency (see Table 2). All black WAS carriers were located with the help of the Immunodeficiency Tumor Registry at the University of Minnesota[14]. Registry of a family with this group was accepted as *prima facie* evidence of the correct diagnosis of WAS in that family.

Eight living women from five families, who had given birth to one or more boys with WAS were contacted either personally or through their primary physician by myself and Ms. Beatrice Spector at the Tumor Registry. The purpose of our study was explained to them and all patients agreed to participate.

II. Screening for G6PD heterozygosity Venous blood samples from each WAS carrier were drawn into EDTA-anticoagulated Vacutainer tubes by the women's primary physicians and reached us by first-class mail within three or four days. A total and differential leucocyte count was made on each sample for future reference. Samples were then pelleted at 450 x g

for ten minutes at 4°C and then washed x3 with phosphate buffered saline (PBS) pH 7.4 under the same conditions. A 50 µl aliquot of washed, packed RBCs was then drawn from the middle of the pellet and mixed with an equal amount of lysis buffer (see under "Preparation of Reagents"). This mixture was then frozen and thawed x3 in a dry ice-acetone bath and electrophoresed on cellulose acetate media as described by Ellis and Alperin[70]. G6PD-specific enzyme staining was done according to the method of Sparkes et al[71] and the proportion of isoenzyme bands estimated visually[57] (see also "G6PD Electrophoresis").

If electrophoresis of RBCs showed only G6PD-B, then the buffy coat was removed from the original RBC pellet, washed, and electrophoresed as described for the RBCs. This procedure allowed us to detect any women who might be $GdA^-/GdB$ heterozygotes since the $A^-$ isoenzyme often loses considerable activity in the RBC but retains >90% activity in the leucocytes[51]. At our request, one patient and her two WAS sons were screened for G6PD type by Drs. John N. Whisnant and H. N. Kirkman at the University of North Carolina at Chapel Hill using similar techniques.

At the beginning of our project we considered using skin fibroblast cultures for obtaining G6PD genotype information since these cells are not of hematogenous origin. Our concern was that, according to our hypothesis, selection against the hematogenous cells carrying the WAS trait in the carrier might possibly have taken place in the erythroid line. If this had indeed happened, then analysis of only hematogenous cells would have given us woefully inaccurate information about the G6PD genotype of true

G6PD heterozygotes. However, both the RBCs and buffy coat from the first patient screened expressed both isoenzyme types, implying that selection, if it had taken place, had not affected the RBCs. Because of this information and the difficulty that most primary physicians might be expected to encounter in attempting a sterile skin biopsy, it was felt that venous blood would serve adequately for screening purposes.

III. WAS carrier heterozygous for polymorphous G6PD As a result of our initial screening efforts only one definite G6PD-heterozygous WAS carrier was found. Patient IV is the 33 year old mother of two children, both boys having the full-blown manifestations of WAS. The eldest son was born in 1969 and has been plagued since birth with thrombocytopenia, chronic otitis media, and oral herpes simplex infections. He has developed chronic lung problems from repeated pulmonary infections. He has also developed a B-cell lymphoma of the bowel and a granulomatous lung mass containing lymphomatous foci, both of which have been surgically resected. The younger son was born in 1974 and has had many of the same problems including thrombocytopenia with hemorrhagic episodes, chronic otitis media, recurrent pneumonias, and severe herpes simplex infections of the face. The mother is normal in every respect. She has no history of recurrent infections, easy bruisability, bleeding problems, or eczema. The remainder of her medical history is unremarkable. Aside from her two WAS sons, she has no other children. There appears to be no family history of infant death or illness similar to WAS, although information on the family of this woman remains very incomplete at this time. Other than our study of G6PD pheno-

types, no other studies have been done on this woman by ourselves or others.

## IV. Preparation of reagents

Washing medium: HBSS+

Hank's Balanced Salt Solution (Flow Laboratories) containing 100 U/ml of potassium penicillin G and 100 μg/ml of streptomycin sulfate (Microbiological Associates).

Lymphocyte medium: RPMI-20%

RPMI-1640 culture medium (Gibco) containing 20% absorbed heat-inactivated fetal calf serum, 100 U/ml of potassium penicillin G, 100 μg/ml of streptomycin sulfate, and 2 mM l-glutamine (Microbiological Associates).

Absorbed heat-inactivated fetal calf serum:

Fetal calf serum (FCS, Gibco) is heat-inactivated for 30 min in a 56°C water bath. One volume of washed, packed sheep red blood cells (SRBC) is then mixed with four volumes of FCS and incubated for two hours at 4°C. The cell suspension is then spun at 800 x g for 10 minutes to remove the SRBC. The serum supernate is then sterilized by ultrafiltration through a 0.22μm Nalgene filter unit.

AET-treated SRBC:

One volume of washed, packed SRBC is added to four volumes of freshly made 0.143 M 2-aminoethylisothiouronium bromide (AET) in distilled water, pH 8.0, and incubated for 15-20 minutes at 37°C. SRBC are then washed x3 in PBS, and then diluted to a concentration of 2% (v/v) with RPMI-20%.

Ficoll-Hypaque solution:

Distilled water is added to 31.77 gm Ficoll-400 (Pharmacia) to make 400 ml of solution. This solution is then mixed with 100 ml of 50% Hypaque solution (Winthrop Laboratories), sterilized by passage through a 0.22 μm Nalgene filter, and stored in the dark at 4°C. Density of the solution is 1.078 g/ml.

Dextran solution:

Dextran (clinical grade, MW 100,000-200,000; ICN Pharmaceuticals) is dissolved in 0.9% NaCl to make a 6% solution.

Phosphate buffered saline (PBS):

3.36 ml of Solution A and 16.00 ml of Solution B is diluted to 1000 ml with 0.9% sterile saline solution to make PBS, pH 7.4. Solution A is made with 34.02 g $KH_2PO_4$ diluted to 500 ml with distilled water. Solution B is made with 87.09 g $K_2HPO_4$ diluted to 1000 ml with distilled water.

Electrophoresis buffer:

0.37 M Tris, 0.004 M disodium-EDTA, and 0.015 M boric acid, pH 9.1. This buffer is made fresh either weekly or after every ten electrophoresis runs, whichever comes first.

Lysis buffer:

Identical to Electrophoresis buffer but in addition, contains 50 μg/ml of nicotinamide adenine dinucleotide phosphate (NADP)(Sigma).

Staining solution:

Ten ml of staining solution containing 2 mg of MTT-tetrazolium, 4 mg of NADP, 10 mg of glucose-6-phosphate, 0.6 ml of a 1 mg/ml phenazine methosulfate solution (prepared fresh), and 9.4 ml of 0.1 M Tris-HCl buffer, pH 8.0 in distilled water is mixed and used immediately. All reagents are from Sigma.

V. Preparation of cell fractions For initial studies, 100-150 ml of venous blood was drawn slowly through a 19 ga butterfly needle into a plastic syringe containing 20-40 U of preservative-free heparin per ml of whole blood, and immediately mixed. Seven ml of venous blood was drawn into EDTA-anticoagulated lavender top Vacutainers for platelet and cell-count studies. By the end of our study complete fractionations with good yield could be carried out on as little as 30 ml of whole blood. Blood samples from the WAS carrier and a normal G6PD heterozygote were coded, packed on ice, and arrived at our laboratory by air freight within 24 hours. Blood samples from normal G6PD heterozygote controls acquired at our laboratory were treated identically except for refrigeration at $4^{\circ}C$ for 24 hours in lieu of the air freight exposure.

Whole blood from the WAS carrier was always processed with a sample from a normal control. The two samples were coded by someone not involved with our project, and then the unknown samples simultaneously

fractionated and electrophoresed as described below. All centrigugations were done in an IEC-PR6000 refrigerated centrifuge at 4°C. Unless otherwise indicated, all cell washes were done at 450 x g for ten minutes. Cells were counted with a Coulter Counter model no. 3037 $Z_F$ and frequently confirmed with manual counts in a hemacytometer counting chamber.

Purity of cell fractions was determined with several methods. Red blood cells (RBC), polymorphonuclear cells (PMN), and mononuclear cell (MN) preparations were Wright-Geimsa stained and subjected to 400 cell differential counts. Platelets were examined under wet mount for contaminating cells. Monocyte/macrophages (M/M) were observed in a hemacytometer and examined for ingestion of carbonyl iron. T-lymphocytes and non-T-lymphocytes were examined for the formation of E-rosettes (a T-cell function) with AET-treated SRBC as described below.

Va. Platelets Platelets were prepared from EDTA-anticoagulated blood by the modified technique of Shapiro et al[34]. Whole blood was first spun at 100 x g for ten minutes to pellet the cells. The top two-thirds of the platelet-rich plasma was then carefully removed and spun at 1000 x g for 20 minutes to pellet the platelets, which were then washed x3 in PBS under the same conditions.

Vb. Erythrocytes The remaining cell populations were purified from the heparinized whole blood samples. Two volumes of this anticoagulated blood was mixed with one volume of 6% dextran solution and the RBCs allowed to sediment at 37°C for 40 minutes[72]. The leucocyte-rich plasma was then removed and saved for the next step. A small aliquot of RBCs

was then removed from the middle of the sedimented cell column and washed x5 in PBS.

Vc. Granulocytes The leucocyte-rich plasma from the previous step was then spun at 450 x g for ten minutes to pellet the leucocytes, which were then washed x3 in PBS to remove the dextran and then resuspended in PBS to a concentration of $5 \times 10^6$ cells per ml. Four ml aliquots of this suspension were then carefully layered onto three ml of Ficoll-Hypaque (FH) solution in 15 ml conical plastic centrifuge tubes (Falcon) and spun at 400 x g for 45 minutes at 4°C according to the method of Böyum[73]. This technique allows separation of granulocytes (PMNs) from mononuclear cells (MNs) by taking advantage of the different densities of these cell groups. After centrifugation the MNs at the interface of the FH and PBS solutions were carefully removed with a sterile pasteur pipet and saved for the next step. The pellet, containing PMNs and a few contaminating RBCs, was washed x2 in PBS to remove the FH solution and was then resuspended in 0.84 % $NH_4Cl$ for ten minutes at room temperature to lyse the RBCs. The remaining PMNs were then washed x5 in PBS.

Vd. Monocyte/macrophages The MN cells from the previous FH interface were washed x3 in HBSS+ to remove the FH solution and then resuspended in RPMI-20% to a concentration of $4 \times 10^6$ cells per ml. This initial MN cell suspension contains a mixture of about 80-90% lymphocytes, 10-20% monocyte/macrophages (M/M), and occasionally rare PMNs which, if present, amount to <0.5% of the total cell population. Two volumes of this cell suspension was then mixed with one volume of carbonyl iron sus-

pension (Lymphocyte Separator Reagent, Technicon Instruments Corp.) and incubated at 37°C on a rotator for 45 minutes[74]. After this incubation, during which the phagocytic M/Ms and rare PMNs have ingested the iron particles, six ml aliquots of the cell/iron suspension were then layered over FH gradients and spun at 400 x g for 20 minutes. The lymphocytes, which are unable to phagocytize the iron particles, remained at the interface and were carefully removed with a sterile pipet and saved. The monocyte/macrophages in the pellet were then carefully washed x2 in HBSS+.

Ve. T-lymphocytes The monocyte-depleted lymphocytes from the previous FH interface were washed x3 with HBSS+ and resuspended to a concentration of $6 \times 10^6$ cells per ml in RPMI-20%. T-lymphocytes were then rosetted with AET-treated Sheep RBCs as described by Kaplan et al[75]. Three ml of lymphocyte suspension, three ml of absorbed FCS, and six ml of 2% AET-SRBC were gently mixed in a 50 ml conical polypropylene centrifuge tube (Falcon) and then incubated for ten minutes in a 37°C water bath. This suspension was then spun at 200 x g for five minutes at room temperature (RT) to pellet the cells and bring the lymphocytes and AET-SRBCs into closer proximity. The tubes were then incubated for ten minutes at RT, mixed gently, again spun at 200 x g for five minutes, and then reincubated for 45 minutes at RT. The supernate was discarded, replaced with RPMI-20%, and the pellet gently resuspended. A small aliquot was mixed with a viability exclusion dye (eosin Y) and the percent rosetted viable lymphocytes determined. If rosetting was adequate, five ml aliquots of this cell suspension were layered onto FH gradients and spun at 900 x g for 20 minutes

at 4°C as described by Wahl et al[76]. This separation takes advantage of the fact that only T-lymphocytes will form rosettes with AET-treated SRBCs, and the fact that rosetted lymphocytes have a higher density than non-rosetted lymphocytes and will thus pass through the FH gradient.

The non-rosetted mononuclear cells at the interface were removed and saved. The cells in the pellet were carefully resuspended and examined for rosetting. If the percent E-rosettes in the pellet was lower than 90-95%, the cell suspension was run over a second FH gradient. The pellets containing >90-95% E-rosetting cells were then resuspended in 0.84% $NH_4CL$ and incubated for ten minutes at RT to lyse the SRBC. The resulting T-lymphocyte suspension was then pelleted and washed x3 in HBSS+.

Vf. Non-T lymphocytes The non-rosetting mononuclear cells from the interfaces in the preceding step were retreated with AET-SRBCs if cell numbers were sufficient, and rerun over FH gradients. The cells remaining at the interface were then washed x3 in HBSS+.

After monocyte depletion with carbonyl iron, the mononuclear cell pool consists essentially of lymphocytes. The lymphocytes removed by E-rosetting are essentially pure T-lymphocytes as the ability to form rosettes with AET-SRBCs is a function found exclusively in that cell line. The remaining non-rosetting lymphocyte pool actually consists of a mixture of B-lymphocytes, null cells (non-B non-T lymphocytes), T-lymphocytes that failed to rosette for some reason, and a very few contaminating monocytes and PMNs that have found their way into this group despite all

the intervening steps designed to eliminate them. The vast majority of these cells are B-lymphocytes. However, since we did not assay this pool for specific B-lymphocyte markers, we prefer to label this pool as non-T (non-rosetting) lymphocytes.

VI. Electrophoresis All fractionated cell samples were pelleted and resuspended in a small amount of lysis buffer and frozen and thawed x3 in a dry ice-acetone bath. Small aliquots (1-2 μl) of each sample were then applied with a fine-point artist's brush to mylar-backed cellulose acetate plates (Sepraphore-X, Gelman Instrument Co.) previously soaked in ice-cold lysis buffer for 30 minutes. The plates were then immediately electrophoresed at 300 V for 40 minutes at 4°C in a Gelman Deluxe Electrophoresis Chamber filled with Tris-EDTA-Borate buffer, pH 9.1, according to the method of Ellis and Alperin[70]. Some of the initial screening samples were electrophoresed on cellulose acetate strips (Sepraphore-III, Gelman) under identical conditions except at 400 for one hour.

After electrophoresis, the separated G6PD isoenzyme bands were made visible by the enzyme-specific staining method of Sparkes et al[71]. Ten ml of stain solution was mixed as described under "Preparation of reagents" and used immediately. Cellulose acetate plates were removed from the electrophoresis unit and immediately immersed in the stain solution for 15 seconds, briefly allowed to drain on a filter paper, and then placed acetate-side down on a glass plate. Excess stain solution was pressed out, the edges sealed with masking tape to prevent drying, and the enzyme bands allowed to develop in the dark for 15 minutes at RT[70]. Cellulose

acetate strips were stained with the filter paper method of Sparkes et al[71].

As mentioned previously, the staining procedure is enzyme-specific. Only human G6PD isoenzymes will precipitate the dye; sheep G6PD (from SRBCs) will not be stained by this procedure. Briefly, the staining reaction works as follows. Through the compounds in the staining solution, G6PD reduces NADP to NADPH, which in turn reduces the yellow water-soluble MTT-tetrazolium dye to a purple water-insoluble formazan dye. Purple bands are thus formed where the G6PD isoenzymes are present on the plate. Hemoglobin often causes non-specific precipitation of formazan dye as well, but this does not interfere with the detection of G6PD because the hemoglobin migrates much slower than the G6PD isoenzymes. Figure 3 illustrates the separation of hemoglobin and these isoenzymes as detected by spectrophotometric scanning, which will be discussed below.

After the plates or strips were developed, they were immediately immersed in a 1% formaldehyde solution for 10-20 minutes to denature the hemoglobin band present in the RBC samples, stop the other enzymatic reactions, and to help preserve the plates. If the hemoglobin was not denatured it would often migrate into the area of the two G6PD isoenzyme bands as the support medium dried.

Two methods were used in an attempt to estimate the isoenzyme ratios in each sample. The first method involved clearing the translucent support media in mineral oil or other solutions to render them transparent for optical scanning. Before scanning could be done, however, the absorption peak of the formazan dye had to be determined. A small amount of precipitated dye

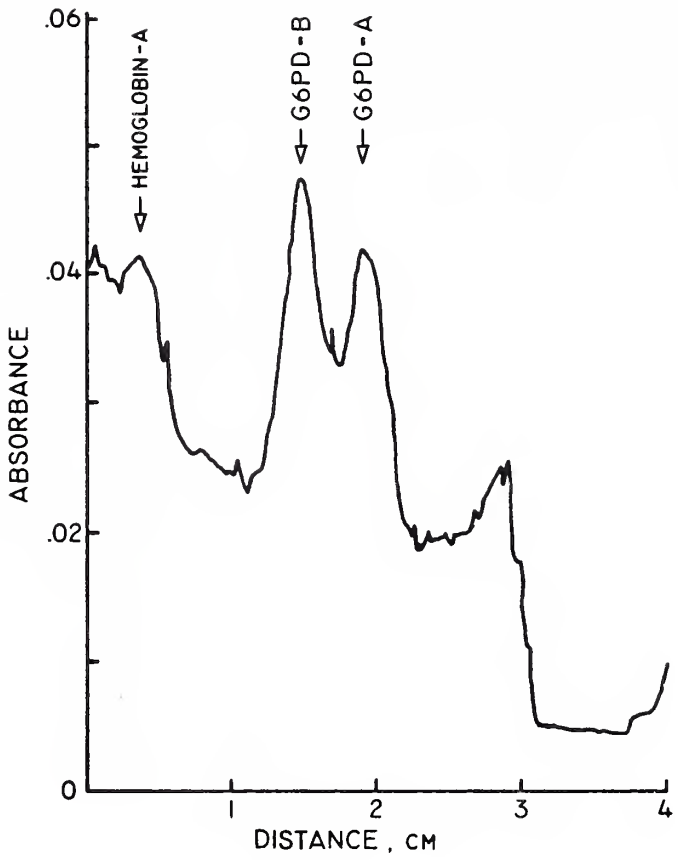


FIGURE 3. Spectrophotometric scan of a representative G6PD isoenzyme electrophoresis.

A hemolysate from a normal heterozygous control was prepared, electrophoresed, and stained as described in the text. The cellulose acetate strip was cleared with mineral oil and scanned at 575 nm in a Beckman DU-2 spectrophotometer with slide scanner.

Note that the G6PD isoenzymes migrate faster than hemoglobin A and that G6PD-A migrates faster than G6PD-B, the distance between the two peaks being about four millimeters. Note also the high background. The origin is not shown on this graph as it was cut away when preparing the cellulose acetate strips for scanning. The abcissa shows relative distance only, not distance from the origin.

was dissolved in methanol, placed in a scanning spectrophotometer, and its absorption spectrum between 350 nm and 750 nm determined (see figure 4). The wavelength of maximum absorption, 575 nm, was then determined from this curve. Once the electrophoresis medium had been cleared it was placed between two glass slides and scanned at 575 nm in a Beckman DU-2 spectrometer with Gifford slide transport. An optical scan of a typical G6PD electrophoresis is shown in figure 3. The areas under each peak were determined with an electronic integrator and the G6PD isoenzyme ratios in each sample calculated from these numbers.

Two major problems with this technique of scanning and G6PD ratio estimating became rapidly apparent. The first was that clearing of the support medium, though necessary for optical scanning, drastically reduced the intensity of the stained bands. The second problem was that the optical scanner was at the extreme limit of its sensitivity. With these two problems combined, we found that bands were occasionally not visualized at all, the background "noise" was quite high, and the two G6PD isoenzyme bands often could not be discriminated as two bands by the machine even though two bands were readily distinguishable by the human eye. For these reasons the mechanical scanning technique was abandoned.

The second method we adopted proved much more reliable. Since the human eye appeared to discriminate two G6PD bands much better than the spectrophotometer we decided to abandon machines altogether. Electrophoresis plates were removed from the formaldehyde solution and, when thoroughly dried, immediately examined on a light-diffuser table. The

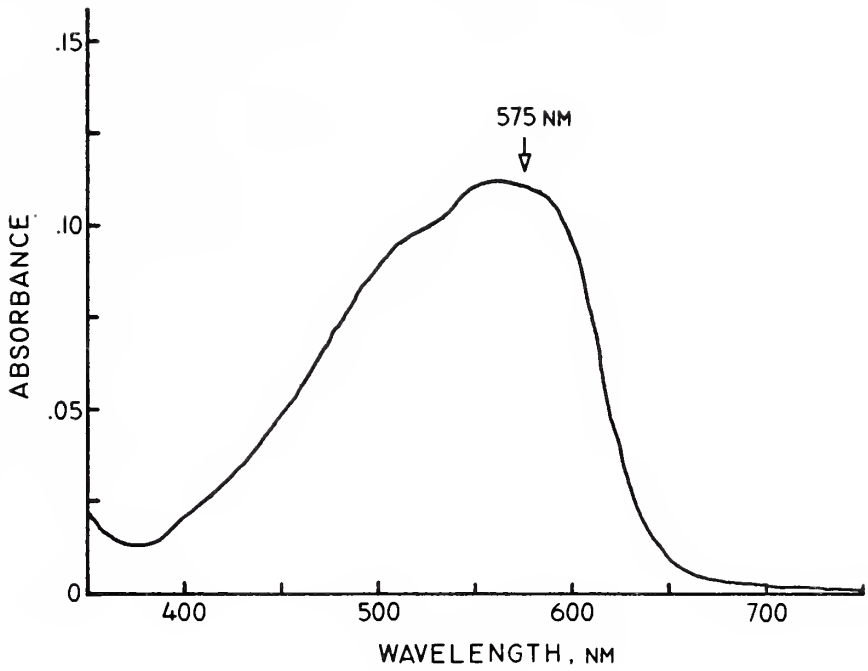


FIGURE 4. Absorbance spectrum of purple formazan dye in methanol.

Yellow MTT-tetrazolium was reduced to the insoluble purple formazan dye which was then pelleted and dissolved in methanol for determination of its absorbance spectrum by scanning spectrophotometry. The visible light wavelengths between 350 nm and 750 nm were scanned. The arrow indicates the 575 nm wavelength used to scan the G6PD electrophoresis strips.

A:B isoenzyme ratio in each sample was then estimated by comparison with known standards. This method allowed us to detect the presence of an isoenzyme fraction making up as little as 5% of the total sample. Photographs of each plate were taken as well, although the sharpness of the bands was much more apparent to the eye than to the camera. After estimation of the G6PD isoenzyme ratios the plates were stored in a notebook in the dark.

## RESULTS

I. Screening Program The outcome of our screening program to locate a G6PD-heterozygous WAS carrier is shown in Table 3. One probable and seven obligate carriers from five different families were tested for G6PD phenotype. As a result of examination of their RBCs, only one definite G6PD heterozygote was found. On electrophoresis, both her RBCs and buffy coat showed 15% G6PD-A. This woman, patient IV, was the only WAS carrier on whom the cell fractionation studies were done. Isoenzyme results on blood samples from her two sons unfortunately were not helpful in determining which G6PD isoenzyme type was linked with the WAS allele. One WAS son was G6PD-A and the other affected boy was G6PD-B, implying a crossover in one of the children.

Three of the women screened appeared to be phenotypically G6PD type B and the remaining four were type A. This latter frequency was much higher than one would expect, considering an AA genotype frequency of 0.09 in black American women (see Table 2). It is most easily accounted for by our very small sample size and the fact that three of the four women were sisters.

Of further interest was the observation that patient V-3a showed only B enzyme in her RBCs and buffy coat despite the fact that her mother, patient V-3, showed only the A isoenzyme. If the mother's genotype was AA, then it is genetically impossible for her daughter's genotype to be BB.

TABLE 3. Results of G6PD isoenzyme phenotype screening in eight black carriers of Wiskott-Aldrich syndrome.

| | G6PD isoenzyme type in: | | |
|---|---|---|---|
| patient # | erythrocytes | buffy coat | WAS son(s) |
| I | B | B | -- |
| II | A | -- | -- |
| III | B | B | -- |
| IV | AB | AB | one A, one B |
| *V-1 | A | -- | -- |
| V-2 | A | -- | -- |
| V-3 | A | A | -- |
| V-3a | B | B | -- |

(--) = not tested

* Patients V-1 through V-3a are all from the same family. V-1, V-2, and V-3 are sisters; V-3a is the daughter of V-3. Note the discrepancy in their G6PD phenotypes, a genetic impossibility. Possible reasons for this are discussed in the text.

Patient IV was the only definite G6PD heterozygote detected and thus was the only patient studied in detail. Of interest is the fact that one of her two affected sons is G6PD-A and the other G6PD-B, implying a crossover. We were thus unable to locate the WAS gene to a specific X-chromosome. See text for further information.

One of these two women must therefore be a heterozygote. There are several possibilities that could explain our apparent inability to detect this. The first is that the heterozygote might be one of the few women who express a large majority of one isoenzyme type. If this type were to make up >95% of the total, the woman would appear to be homozygous because of the 5% sensitivity threshold of our G6PD assay. Another possibility could be that the mother is really genotype $A^-A$ and her daughter $A^-B$. The mother would show only A on electrophoresis and it might be possible to miss a faint $A^-$ band in the daughter. However, the daughter's buffy coat showed only a B band, and it should be remembered that the $A^-$ enzyme retains >90% of normal activity in nucleated cells. In addition, the mother's RBCs showed a normal G6PD staining density after electrophoresis, a crude measurement of enzyme activity implying that she does not have substantial $A^-$ enzyme. A third possibility is that the daughter could be heterozygous for G6PD in normal tissue such as skin fibroblasts, but hemizygous in her RBCs and leucocytes as a result of a selection process that we have hypothesized. However, we chose not to examine skin fibroblasts since the first patient screened (patient IV) had heterozygous RBCs. This of course does not rule out selection in the RBCs of patient V-3a but no selection in the RBCs of patient IV. These possibilities will be discussed in depth later. A fourth possible cause of this discrepancy is the presence of a mutant form of G6PD in one of these women, although these are quite rare. The answers to the puzzle that this family presents unfortunately must await further scrutiny of non-hematogenous tissues in these women, as well as a de-

tailed analysis of the G6PD pedigree in the entire family.

II. Cell Fractionation Samples of whole blood from four normal controls and two separate preparations from our WAS carrier were fractionated into RBCs, platelets, granulocytes, monocyte/macrophages, T-lymphocytes, and non-T lymphocytes. The purity of each cell fraction is summarized in Table 4. Purity of RBCs and platelets was virtually 100%, with less than one contaminating cell per 10,000 RBCs or platelets. The platelet samples from control 4 and our second carrier preparation, pt. IV(#2), rather than being prepared from 24 hour old blood, were isolated immediately after removal from the patient by pelleting from platelet-rich plasma as described previously. This unwashed pellet was then sent to us on ice with the whole blood samples. The platelet-rich plasma prepared from pt. IV(#2) was reported to have shown slight hemolysis. Some of this was carried over in the unwashed platelet pellet for when the platelets were prepared for electrophoresis, a very slight trace of hemoglobin was noted. However, the presence of this hemolysis (and possible contaminating G6PD isoenzymes) did not affect the results of isoenzyme ratio determination, as shown below.

The purity of granulocyte (PMN) preparations from control samples drawn in our lab was routinely >93%, the contaminating cells being mostly mononuclear cells with a few RBCs that would not lyse with ammonium chloride. The poor granulocyte purity in control 4 and the two WAS carrier preparations was due to the quality of blood samples shipped by air freight. Although adequately heparinized and handled impeccably, a fine fibrin clot-

TABLE 4. Purity of representative cell fractions separated from whole blood of controls and one WAS carrier.

| | percent of desired cell type in each fraction | | | | | percent E-rosettes | | |
|---|---|---|---|---|---|---|---|---|
| | RBCs | platelets | PMNs | mono-nuclear cells | monocyte macro-phages | total lymphocyte pool | T-cells | non-T lymphos |
| control 1 | 100 | 100 | 98.8 | 100 | 89.9 | 85.4 | 95.0 | 10.4 |
| control 2 | 100 | 100 | 93 | 99.5 | -- | 64.7 | 96 | 8 |
| control 3 | 100 | 100 | 94 | 93 | 96 | 47.7 | 93 | -- |
| control 4 | 100 | 100 | 56.8 | 91.3 | -- | 72 | 94.5 | 4 |
| pt. IV (#1) | 100 | 100 | 43.7 | 94.7 | 78 | 44.3 | 93.5 | 8 |
| pt. IV (#2) | 100 | 100* | 59.2 | 93.3 | -- | 65.2 | 91.5 | 5.3 |

* Slight hemolysis was present, see text for further explanation. (--) = not done

Samples were prepared and assayed for purity as described in the text. RBCs = red blood cells, PMNs = granulocytes, T-cells = T-lymphocytes.

like material was present in all three samples on arrival in our laboratory. Although the etiology of this problem remains unknown, we feel that its presence in both control and WAS carrier samples indicates that it is most likely a result of technique rather than a defect intrinsic to the WAS carrier. This clot-like material was not observed in samples from controls 1-3, which were drawn in our lab but treated identically. These control samples sedimented normally with dextran, and cell fractionation was accomplished without problems. The presence of the clot-like material in the samples from control 4 and our WAS carrier, however, made dextran sedimentation impossible and granulocytes had to be prepared from the buffy coat; a procedure which gave poorer yield and more RBC contamination than the dextran sedimentation method. This clot also caused the leucocytes to clump and thus separate poorly through the FH gradients. As a result, granulocyte and mononuclear cell fractions had to be passed over several FH gradients to obtain usable purity, even though this greatly diminished the yield. Despite these efforts, granulocyte samples from these patients remained heavily contaminated with mononuclear cells.

In contrast, however, mononuclear cell fraction purity was quite high, usually >92%. Contaminating cells were usually granulocytes and RBCs since ammonium chloride lysis had not yet been done. The granulocytes present in this fraction were a potential problem, since the next step in purification was monocyte/macrophage isolation by carbonyl iron ingestion. Granulocytes are able to phagocytize iron particles as well as the monocytes, and these cells are thus separated together by our technique. Purity of the

monocyte/macrophage pool was judged by the presence or absence of iron particles in viable cells rather than by monocyte-specific histochemical staining. It is therefore possible that the monocyte/macrophage pool contained undetected granulocytes, although the total mononuclear cell pool was substantially granulocyte-free prior to the carbonyl iron isolation.

As can be seen in Table 4, the purity of lymphocyte fractions was excellent. T-lymphocytes were >92% pure by E-rosette formation whereas the non-T lymphocytes were substantially rosette-free. The cells comprising this latter cell pool were mostly B-lymphocytes. Other mononuclear cells potentially present in this preparation include null cells (non-B non-T lymphocytes), T-lymphocytes that did not rosette, and monocytes that did not ingest iron. Since B-lymphocyte-specific immunofluorescent staining was not done on these samples however, the exact percentage of B-lymphocytes found in them is unknown. We have therefore chosen to classify this group as non-T lymphocytes.

Results of the G6PD phenotype determination on lysates of the various cell fractions from controls and our WAS carrier are summarized in Table 5. The ratio of G6PD isoenzymes in all the normal controls was remarkably constant from cell line to cell line within the same individual. In addition, RBCs, granulocytes, monocytes, and non-T lymphocytes from the WAS carrier showed similar G6PD ratios. In contrast to the pattern seen in normal controls however, the platelets and T-lymphocytes from our WAS carrier showed a complete absence of the A enzyme on multiple electrophoreses. This absence of the A enzyme in these

TABLE 5. G6PD isoenzyme ratios in different cell fractions isolated from whole blood of three normal heterozygous controls and one doubly heterozygous WAS carrier.

| | (percent G6PD-A) : (percent G6PD-B) in each cell fraction | | | | | |
|---|---|---|---|---|---|---|
| patient | RBCs | platelets | granulocytes | monocyte-macrophages | T-lymphocytes | non-T lymphocytes |
| control 2 | 50:50 | 50:50 | 50:50 | 50:50 | 50:50 | 50:50 |
| control 3 | 60:40 | 60:40 | 65:35 | 60:40 | 60:40 | 60:40 |
| control 4 | 55:45 | 55:45 | 60:40 | -- | 60:40 | 55:45 |
| pt. IV (#1) | 15:85 | 0:100 | 20:80 | 15:85 | 0:100 | 10:90 |
| pt. IV (#2) | 15:85 | 0:100* | 15:85 | -- | 0:100 | 15:85 |

* despite slight hemolysis, see text. (--) = not done

Cell lysates were electrophoresed and G6PD isoenzyme ratios determined as explained in the text. Points to note are the remarkable constancy of isoenzyme ratios from cell fraction to cell fraction within the same individual control patient, and the complete absence of G6PD-A in the platelets and T-lymphocytes of the WAS carrier on two separate occasions. The significance of this absence is explained in the text.

samples was felt to be the result of a process within the patient rather than a result of our technique, since the pattern was seen in two different cell separations done several months apart. Furthermore, the A band in control samples was always visualized when these samples were electrophoresed simultaneously with the platelet and T-cell fractions from our WAS carrier.

Despite the 56% contamination by lymphocytes in one granulocyte sample and the 41% contamination in the second sample from the carrier, it was felt that the 15:85 isoenzyme ratio in her granulocytes was reliable for two reasons. The first was that A:B ratios in the buffy coat preparations from the WAS carrier matched the 15:85 ratio seen in her RBCs. If her granulocytes had been missing the A enzyme, this, combined with the complete absence of the A enzyme seen in her T-lymphocytes, would have resulted in a buffy coat ratio much lower than the 15:85 actually seen. The second reason involves similar calculations since our contaminated granulocyte preparations resemble a buffy coat in many ways. If pure granulocytes were actually missing the A enzyme, but were 50% contaminated with cells expressing 15% A (the largest amount of A enzyme any of her cells can express), then we should see an electrophoresis pattern containing only 7.5% A enzyme. In addition, if half of the contaminating cells were T-lymphocytes (a reasonable estimate) we would expect to see a granulocyte pattern showing less than 5% A enzyme. Despite similar amounts of contamination however, the actual granulocyte preparations from our carrier still showed 15% A.

Since this is the pattern found in her RBCs and other heterozygous cell lines of higher purity than the granulocytes, we can only conclude that the granulocytes of our carrier were heterozygous as well, despite contamination.

The purity of the remaining cell fractions was sufficiently high such that their electrophoresis patterns were felt to be an accurate reflection of their G6PD phenotype. To summarize our results, cell fractions from normal controls showed remarkably constant G6PD isoenzyme ratios from cell line to cell line. In contrast, cell fractions from a WAS carrier showed constant isoenzyme ratios in RBCs, granulocytes, monocyte-macrophages, and non-T lymphocytes; but complete absence of the A enzyme in platelet and T-lymphocyte pools. The possible causes of this pattern and its significance will be discussed below.

## DISCUSSION

As mentioned previously, the apparent discrepancy between the G6PD genotype of our WAS carrier and the G6PD phenotype of her platelets and T-lymphocytes is not methodological. Our results are most unusual since similar hemizygous G6PD phenotypes have been seen only in monoclonal tumor cell lines[62,63] and in carriers of the X-linked disorders of HPRT deficiency[22] and abnormal phosphoribosyl-pyrophosphate synthetase (PRPPS) activity[68]. Our WAS carrier was not ill and is presumed not to be a carrier for the latter two disorders since their occurrence is quite rare and there was no evidence that her two sons express those disorders in addition to WAS. Furthermore, RBCs from carriers of these latter two disorders show hemizygous G6PD expression, whereas the RBCs from our WAS carrier were heterozygous.

Several mechanisms for this apparent hemizygous expression in our WAS mother are possible. Let us first consider her platelets only. If, as mentioned earlier, the megakaryocytes from a WAS carrier were a mosaic population with 50% making "defective" platelets and 50% making normal platelets, one might still expect only 10% of the total peripheral platelet pool to have originated from WAS megakaryocytes because of production and destruction dynamics. Thus even though most of her cell lines would have a G6PD isoenzyme ratio of 50:50, one would see a ratio of 10:90 in her platelet pool. However, our

WAS carrier appears to be a 15:85 mosaic rather than 50:50. If the former ratio was also seen in her megakaryocytes, with 15% producing abnormal platelets and 85% producing normal platelets, then by extrapolation one might expect <4% abnormal platelets in the peripheral platelet pool. This pool would then have a G6PD ratio of less than 4:96 but we would be unable to tell the difference between this and a ratio of 0:100 since the sensitivity threshold of our G6PD assay for one isoenzyme component in a heterozygote is 5%. The hemizygous result found in the platelet pool of our WAS carrier might therefore be a result of platelet dynamics combined with her uneven Lyonization.

This model however, cannot explain the hemizygous findings in T-lymphocytes so we must therefore consider additional ideas. The hemizygous G6PD expression in both T-lymphocytes and platelets in our WAS carrier are most likely the result of a genetic event involving bone marrow stem cells or their hematogenous progeny. A number of genetic mechanisms could be responsible for these findings, including recombination, somatic mutation, or deletion. However, in the case of the X-chromosome it seems most likely that the mechanism involves X-inactivation. It appears, at least in the T-lymphocytes and possibly the platelets or megakaryocytes of the WAS carrier, that either X-chromosome inactivation is not random, or that following random inactivation there is competition and selection against one of the two cell lines. The most appealing and intriguing theory is that selection takes place

against the megakaryocytes and T-lymphocytes expressing the WAS allele because of some intrinsic defect in those cells. Unfortunately we cannot tell whether the hemizygous cell lines carry the normal allele or the WAS allele since one WAS son expresses G6PD-A and the other WAS son expresses G6PD-B, implying a crossover in one of the children. Despite this missing bit of evidence, we still feel that selection against cells bearing the WAS allele has taken place in the hemizygous cell lines of our WAS carrier.

The theory of selection in our WAS carrier is supported by studies in several other X-linked disorders in which selection appears to have taken place against cell lines bearing the defective allele. For example, Nyhan et al[22] were intrigued by the observation that, in the carriers of Lesch-Nyhan disease (HPRT deficiency), fibroblast cultures showed HPRT activity intermediate between normals and affected boys (as expected), but RBCs and lymphocytes from carriers appeared to show full normal activity. To investigate this discrepancy they found a woman doubly heterozygous for both HPRT deficiency and polymorphous G6PD. They found, as had others, that fibroblast cultures from this woman were made up of two clones, one showing full HPRT activity and expressing G6PD-B, and the other clone showing absent HPRT activity and expressing G6PD-A. However, studies on her RBC and leucocyte pools showed only G6PD-B. The complete absence of the population of cells expressing G6PD-A and HPRT deficiency would of course explain why the blood cell pools had full normal activity rather than

the intermediate activity seen in the heterozygous fibroblast cultures. Nyhan et al concluded that there was selection against the HPRT deficient stem cells in the bone marrow resulting in the hemizygous expression of G6PD-B and normal HPRT in BCs and leucocytes from the carrier. To our knowledge, they have not assayed each separate leucocyte population to see if all hematogenous cell lines are hemizygous or whether this is limited to one or two specific cell lines.

A similar finding was seen by Yen et al[68] when studying the X-linked inheritance patterns of phosphoribosylpyrophosphate synthetase (PRPPS) in families with a mutant form of the enzyme. This mutant enzyme had a different electrophoretic mobility than the normal enzyme, and in addition had increased enzyme activity rather than the decreased activity usually seen in hereditary disease states. This group found that fibroblasts from carrier mothers showed PRPPS activity intermediate between normals and affected males and that cultures of these fibroblasts, just as in Lesch-Nyhan carriers, were comprised of two clones, one with normal PRPPS activity and electrophoretic mobility, and the other clone expressing the elevated activity and electrophoretic mobility seen in affected males. Erythrocyte and purified lymphocyte pools, however, did not display the mixture of PRPPS enzymes seen in fibroblast pools, but rather, the full elevated activity and electrophoretic pattern of the mutant enzyme. The presence of only the mutant enzyme in these hematogenous pools resembled the findings in the carriers of Lesch-Nyhan disease. However, Yen et al felt that competition in the

stem cells favored selection of the mutant cells expressing increased PRPPS activity. They also reported that another group[69] had made similar observations in a different family with increased PRPPS activity. Selection in the carrier from this family appears to have favored the normal enzyme. Yen et al therefore concluded that the hemizygous RBCs and lymphocytes in the carriers of mutant PRPPS activity was more likely a result of selection rather than non-random X-inactivation since a different isoenzyme appeared to be favored in each of the two cases.

The results of our experiment and the supportive evidence of these other X-linked models suggest that there is either non-random X-inactivation in megakaryocytes and T-lymphocytes or that selection against one of the X-chromosome clones has taken place in these cell lines. However, because of the particular cell lines that appear to be hemizygous, we believe that this process is one of selection rather than non-random inactivation. Let us assume for the moment that either event could occur in our WAS carrier. This event cannot have taken place at the level of the pluripotent stem cell since the RBC, granulocyte, monocyte, and non-T lymphocyte pools are heterozygous. Because T-lymphocytes are hemizygous and non-T lymphocytes are not, it appears that inactivation or selection has taken place after the lymphocyte precursors have migrated through the thymus, which first takes place during the seventh or eighth week of gestation[77]. However, Migeon and Kennedy[56] and Fialkow[57] have determined that random

X-inactivation is definitely complete by the fourth week of gestation and probably occurs as early as the 16 cell blastula. If our hemizygous pattern was a result of non-random X-inactivation taking place at this stage of embryonic development, then all lymphocytes should be hemizygous since the thymus has not yet developed. In our WAS carrier however, only the thymus-derived lymphocytes are hemizygous, implying that the event took place after development of the thymus. Since the timing of this event is clearly later than the appearance of X-inactivation, we submit that the hemizygous pattern in T-lymphocytes and platelets can only be a result of selection against cells bearing a particular X-chromosome.

As mentioned before, which particular X-chromosome has been selected against is unknown. If, however, the WAS lesion involves a defect in or inhibitor of the Krebs cycle and subsequent defective energy production as has been postulated by Shapiro et al[34], then the cells expressing this defect in the heterozygous carrier might certainly be at a competitive disadvantage. We feel therefore that the cells expressing the WAS allele are the most likely to be the ones missing from the T-lymphocyte and platelet pools.

The hemizygous platelet pool can be explained as resulting from either platelet dynamics or actual selection against one of the megakaryocyte pools, but the hemizygous T-lymphocytes can only be explained by selection. We feel it is most likely that both hemizygous groups are a result of selection, and that this process was directed against

the WAS allele. However, it should be remembered that the heterozygous cell lines in this mother still carry the WAS gene. It is certainly reasonable to expect that selection would take place only in a cell line expressing the WAS allele, but whether all cell lines expressing this allele have been selected against is unknown. In other words we don't know for sure that expression of the WAS allele in carriers is limited only to the platelets (megakaryocytes) and T-lymphocytes. If this was the case however, it could certainly be one explanation as to why the carriers manifest no intermediate form of the disorder seen in their affected sons.

Let us now compare our anti-WAS selection theory with the platelet data in carriers. The platelet size data of Murphy et al[32] indicate that all platelets in the carrier are of normal size. On page 33 of this thesis three possible explanations were considered. The first was that the megakaryocytes were a mosaic (no selection) but that abnormal platelets were destroyed so fast that their presence could not be detected by Murphy's technique. The platelet pool would thus appear hemizygous. Although we have no information about the megakaryocytes themselves, the platelets in our carrier fit this picture and this theory is still possible.

Again assuming no selection in megakaryocytes, the second explanation for the absence of abnormally small platelets was that the normal cells produced some positive "factor" enabling WAS megakaryocytes to produce normal platelets. Though of normal size, the platelets would

be genetically heterozygous. Our finding of hemizygous platelets contradicts this theory.

The third explanation was that selection had taken place, leaving only normal megakaryocytes producing a normal hemizygous platelet pool. Our data support this theory of selection as well as the platelet dynamics theory. The only way to determine which is correct is to study the clonal patterns in the megakaryocytes themselves, for in the first model these cells will be heterozygous while in the selection model they will be hemizygous.

Regardless of which model is correct, one can infer from our data that the vast majority, if not all, of the platelets in the peripheral blood of our carrier are of monoclonal origin. Murphy's data indicate that these platelets are of normal size and thus they have probably originated from normal megakaryocytes. How then can one explain the physiological defects reported by Baldini[27], Kuramoto et al[25], and Shapiro et al[34]? Three explanations taking all of this into account were proposed earlier. The first was that megakaryocytes were a mosaic (no selection) but that normal cells exerted some "positive influence" causing them to produce normal-sized platelets and therefore a heterozygous platelet pool. The WAS cells then exerted a "negative influence" on all the platelets, causing the abnormal physiologic responses. This theory was quite awkward, but is definitely contradicted by our platelet findings.

The second theory involved megakaryocyte selection resulting in

a normal hemizygous platelet pool upon which a "negative influence" (perhaps a Krebs cycle inhibitor produced by other WAS cells) results in abnormal physiologic responses. Our findings support the idea of a normal platelet pool, and the existence of a soluble inhibitor is certainly possible. However, if selection has occurred in all cell lines expressing the WAS allele, from where could this proposed factor originate?

The third explanation was that the physiologic defects seen in the platelets of carriers were an artifact and thus do not actually exist. From our data and that of Murphy et al it is quite clear that the platelet pool in these carriers is essentially of normal origin. The proposed abnormal platelet responses are thus very intriguing for there is much to suggest that they should not exist, yet they do appear to be present in some cases. They remain a puzzle to say the least.

One of our reasons for initiating our study of the WAS carrier state was to see if we could locate the cell line responsible for the immune defect in the affected sons. Almost all of the hematogenous cells have been implicated at one time or another but no actual defect has been found in any of them. Is it possible to locate this defect in the sons by studying their mothers? We must make several assumptions in order to attempt this. First, we do not know if the apparent selection we see in the carrier has occurred against the WAS allele or the normal allele, although we suspect the former. Assuming this, we still do not know if there is selection against all cells expressing the WAS allele

in the mother. We also do not know whether the cells selected against in the carrier are the same cells that are responsible for the defect in their affected sons. At the very least, we could assume that if a WAS cell were defective in the mother it should be defective in the son.

Understanding full well that our assumptions are unproven, we would like to make the intuitive leap and suggest that the hemizygous T-lymphocytes found in the mother implicate the T-lymphocyte as at least one if not the only cell responsible for the immune defects seen in the affected males. This notion has already been supported to some extent by the bone marrow transplantation work of Parkman et al[12] as discussed earlier. The mechanism by which the T-cell could produce these defects is unknown although it may lie in the inability of the T-lymphocyte to recognize the immune message presented to it by any of several methods. The other cell line that is frequently suggested as the cause of the defects in afferent immunity in this disorder are the monocytes which play a large role in processing and presenting antigens to the T-lymphocyte. Because of our results in the mother showing that her monocyte/macrophage pool was heterozygous we feel that the monocyte/macrophage in the boys is not responsible for the immune defects.

In addition to the suggestions about the basis for the immune defect in the sons, our results in the carrier mother suggest a possible method for detecting the carrier state. If all carriers indeed express hemizygous platelet and T-lymphocyte pools with respect to the X-chromo-

some then detection of the carrier state would involve assaying one of these cell lines for the hemizygous expression of a commonly heterozygous X-linked marker. Isolation of purified platelets is quite easy, and isolation of T-lymphocytes is fairly uncomplicated as well. However, the most common X-linked marker currently known is G6PD, which is heterozygous in only 42% of black women. Thus roughly half of the black women suspected as being carriers could be detected, but the techique is useless for the majority of the population who are homozygous for this marker. Widespread application of this hemizygous detection technique will have to await the discovery of other more common heterozygous markers (either structural or genetic) on the X chromosome.

We should hasten to point out several problems. Because of the apparent crossover in one of our carrier's WAS sons, we were unable to locate the WAS trait to a particular chromosome. In addition we have studied only one carrier and do not know whether our observations were a freak occurrence or whether they are found in all carriers of Wiskott Aldrich syndrome. The clonal status of the megakaryocytes in our carrier is also unknown. Studies are currently underway to locate and test more doubly heterozygous WAS carriers, however, and we feel that evidence to support our theories will be shortly forthcoming.

The technique of using genetic markers has been widely used in the study of tumors and hematological disorders, but to our knowledge we are the first to apply it to the study of the immune system. This

preliminary study has been useful in understanding some of the aspects of Wiskott-Aldrich syndrome and may indeed prove useful in understanding the other X-linked disorders of immunity such as X-linked immunodeficiency with hyper-IgM, X-linked infantile hypogammaglobulinemia, and the X-linked form of severe combined immunodeficiency. One major drawback is the extreme rarity of these diseases, and the even more remote possibility of finding a carrier heterozygous for a studiable X-linked marker. Our work however, serves as a model for the application of this technique to the understanding of immune disease and the normal immune system.

## SUMMARY

In an attempt to locate the cell lines responsible for the immune defects in boys with the X-linked Wiskott-Aldrich syndrome, the X-inactivation patterns of hematogenous cell lines in the heterozygous carriers were studied with the use of glucose-6-phosphate hehydrogenase (G6PD) as a genetic marker. One probable and seven obligate carriers from five black families were screened for G6PD genotype and one heterozygote was detected. Erythrocytes, granulocytes, monocyte/macrophages, and non-T lymphocytes showed identical G6PD isoenzyme ratios. However, the platelets and T-lymphocytes from this carrier showed the complete absence of the A isoenzyme. This pattern was not seen in normal controls, who showed a remarkable consistency of isoenzyme ratios in all cell lines. We feel that the hemizygous platelets and T-lymphocytes found in the carrier result from selection against those cells bearing the Wiskott-Aldrich allele and that by inference the T-lymphocyte is at least partially responsible for the immune defects seen in the affected males. A possible method for detection of the carrier state, as well as the application of this chromosome marker technique to other X-linked immunologic diseases is discussed.

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